

**REG-LIKE PROTEIN IMMUNOGLOBULIN DERIVED PROTEINS,
COMPOSITIONS, METHODS AND USES**

BACKGROUND OF THE INVENTION

5 FIELD OF THE INVENTION

The present invention relates to human Ig derived proteins (Ig derived proteins),
specified portions or variants specific for at least one REG-Like Protein (RELP) protein or
fragment, RELP protein immunoglobulin derived protein encoding and complementary nucleic
acids, host cells, and methods of making and using thereof, including therapeutic formulations,
10 administration and devices.

RELATED ART

Tumors are abnormal masses of tissue. When tumors proliferate uncontrollably, they
are said to become malignant. This condition is generally referred to as a cancer. Numerous
15 methods are used to determine when a patient has developed a tumor and when the condition
has become cancerous. The identification or quantitation of various tumor or cancer markers
is one desirable means for making such determinations.

Broadly, a "marker" is any property that can be used to distinguish cancer from normal
tissues and from other disease states. The markers' presence is then a basis for classification.
More specifically, the term is used to denote particular molecules that are amenable to assay.
20 Serum markers, as the name implies, are markers that are readily assayed in the serum of a
patient. Typically, they are secreted proteins or cell receptors that are abundant in tumor cells
well beyond their presence (or total absence) in normal cells and tissues. Examples include
PSA, CEA, and AFP.

A more expansive consideration of tumor and cancer markers includes the detection of
25 tumors and cancer from the nucleic acids produced in various cells (as well as other materials
that are related to nucleic acids). Cancer is generally considered to be a disease of multiple
mutations. Thus, detection of the mutations at the molecular level offers the prospect of more
direct and more reliable diagnoses than was possible with some of the older cancer markers.
30 Thus, it is appropriate to consider a nucleic acid sequence that is indicative of the mutation that
causes or occurs with the cancerous condition to be a cancer marker. The ability to conduct
nucleic acid analyses does not vitiate the value of serum markers, however. Each may have an
appropriate role to play in the diagnosis, staging, and treatment monitoring of a patient.

Discovering genes that encode cancer-associated antigens and events also opens the door to genetic intervention against cancer cell proliferation. The accurate and consistent use of a cancer marker to differentiate cancerous from normal tissue, not only has diagnostic potential, but is also desirable for treatment and prognosis. Therefore, such markers continue
5 to be sought.

The reg proteins, which belong to the C-type lectin superfamily, are secreted proteins of about 20 kD in size. They are found in normal and malignant tissues of the gastrointestinal tract, in the pituitary and in regenerating neurons. Reg expression associates with cell proliferation, migration and differentiation (Chiba T et al., 2000, J Gastroenterol 35 Suppl
10 12:52, Levine JL, 2000, Surg Res 89:60, Otonkoski T et al., 1994, Diabtets 43:1164, Bernard-Perrone FR, 1999, J Histochem Cytochem 47:863). The known reg genes cluster on human chromosome 2p12.

The first characterized member of the reg protein family was Reg1, which was isolated from rat regenerating pancreatic islets (Terazono et al., 1988). Subsequently, cDNAs encoding for four additional human reg proteins, and the corresponding mouse and rat orthologs, have been cloned (Watanabe et al., 1990; Lasserre et al., 1992; Bartoli et al., 1993; Rafaeloff et al., 1997). They exert mitogenic activity to subsets of epithelial and neuroectodermal cells (Katsumata et al., 1995, Zenilman et al., 1996; 1997; 1998; Livesey et al., 1997). A growth signal transducing receptor for rat reg1 proteins was recently described. The receptor is encoded by a gene homologous to human multiple exostoses gene. It was found to have been expressed, in addition to pancreatic islets, in various tissues including kidney, liver, gut, the adrenal and pituitary glands (Kobayashi S et al. 2000).

Identification, isolation, and use of new tumor and cancer markers remain important in the diagnosis, treatment and prevention of cancer.

Accordingly, there is a need to provide RELP protein human antibodies or specified portions or variants, nucleic acids, host cells, compositions, and methods of making and using thereof, that overcome one more of these problems.

SUMMARY OF THE INVENTION

The present invention provides isolated RELP protein human Ig derived proteins (Ig derived proteins), including immunoglobulins, cleavage products and other specified portions and variants thereof, as well as RELP Ig derived protein compositions, encoding or complementary nucleic acids, vectors, host cells, compositions, formulations, devices,

transgenic animals, transgenic plants, and methods of making and using thereof, as described and enabled herein, in combination with what is known in the art.

The present invention also provides at least one isolated RELP Ig derived protein or specified portion or variant as described herein and/or as known in the art.

5 The present invention provides, in one aspect, isolated nucleic acid molecules comprising, complementary, or hybridizing to, a polynucleotide encoding specific RELP Ig derived proteins or specified portions or variants thereof, comprising at least one specified sequence, domain, portion or variant thereof. The present invention further provides recombinant vectors comprising said isolated RELP Ig derived protein nucleic acid molecules,
10 host cells containing such nucleic acids and/or recombinant vectors, as well as methods of making and/or using such Ig derived protein nucleic acids, vectors and/or host cells.

At least one Ig derived protein or specified portion or variant of the invention binds at least one specified epitope specific to at least one RELP protein, subunit, fragment, portion or any combination thereof. The at least one epitope can comprise at least one Ig derived protein binding region that comprises at least one portion of said protein, which epitope is preferably comprised of at least one extracellular, soluble, hydrophilic, external or cytoplasmic portion of said protein.
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The at least one Ig derived protein or specified portion or variant can optionally comprise at least one specified portion of at least one CDR (e.g., CDR1, CDR2 or CDR3 of the heavy or light chain variable region) and/or at least one framework region. The at least one Ig derived protein or specified portion or variant amino acid sequence can further optionally comprise at least one specified substitution, insertion or deletion.
20

The present invention also provides at least one composition comprising (a) an isolated RELP Ig derived protein or specified portion or variant encoding nucleic acid and/or Ig derived protein as described herein; and (b) a suitable carrier or diluent. The carrier or diluent can optionally be pharmaceutically acceptable, according to known methods. The composition can optionally further comprise at least one further compound, protein or composition.
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The present invention also provides at least one method for expressing at least one RELP Ig derived protein or specified portion or variant in a host cell, comprising culturing a host cell as described herein and/or as known in the art under conditions wherein at least one RELP Ig derived protein or specified portion or variant is expressed in detectable and/or recoverable amounts.
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The present invention further provides at least one RELP Ig derived protein, specified

portion or variant in a method or composition, when administered in a therapeutically effective amount, for modulation, for treating or reducing the symptoms of, at least one malignant disorder or disease in a cell, tissue, organ, animal or patient and/or, as needed in many different conditions, such as but not limited to, prior to, subsequent to, or during a related

5 disease or treatment condition, as known in the art and/or as described herein.

The present invention also provides at least one composition, device and/or method of delivery of a therapeutically or prophylactically effective amount of at least one RELP Ig derived protein or specified portion or variant, according to the present invention, optionally further comprising a cytotoxic or chemotherapeutic agent suitable to killing or substantially

10 inhibiting the growth of an RELP-containing abnormal or malignant cell or tissue, in vitro, ex vivo or in vivo.

DESCRIPTION OF THE FIGURES

15 FIG. 1 is the nucleic acid sequence of the cDNA that encodes for RELP (Seq. ID No. 1).

FIG. 2 is the amino acid sequence of RELP (Seq. ID No. 2).

20 FIG. 3 is the nucleic acid sequence of the cDNA that encodes for RELP signal protein (Seq. ID No. 3). FIG. 3a is the amino acid sequence of RELP signal protein (Seq. ID No. 4).

FIG. 4 is a scaled schematic representation of the RELP gene.

25 DESCRIPTION OF THE INVENTION

The present invention provides isolated, recombinant and/or synthetic RELP Ig derived proteins or specified portions or variants, as well as compositions and encoding nucleic acid molecules comprising at least one polynucleotide encoding at least one RELP Ig derived protein. Such Ig derived proteins or specified portions or variants of the present

30 invention comprise specific full length Ig derived protein sequences, domains, fragments and specified variants thereof, and methods of making and using said nucleic acids and Ig derived proteins or specified portions or variants, including therapeutic compositions, methods and devices.

As used herein, a "REG-Like Protein Ig derived protein," "RELP Ig derived protein," "RELP Ig derived protein portion," or "RELP Ig derived protein fragment" and/or "RELP Ig derived protein variant" and the like decreases, blocks, inhibits, abrogates or interferes with RELP protein activity, binding or RELP protein receptor activity or binding *in vitro*, *in situ* and/or preferably *in vivo*. For example, a suitable RELP Ig derived protein, specified portion or variant of the present invention can bind at least one RELP protein and includes anti-RELP Ig derived proteins, antigen-binding fragments thereof, and specified portions, variants or domains thereof that bind specifically to RELP protein. An RELP Ig derived protein of the present invention optionally further comprises a suitable toxic or chemotherapeutic agent. A suitable RELP Ig derived protein, specified portion, or variant can also decrease block, abrogate, interfere, prevent and/or inhibit RELP protein RNA, DNA or protein synthesis, RELP protein release, RELP protein receptor signaling, membrane RELP protein cleavage, RELP protein activity, RELP protein production and/or synthesis.

Anti-RELP Ig derived proteins (also termed RELP Ig derived proteins) useful in the methods and compositions of the present invention are optionally characterized by high affinity binding to RELP protein and optionally having low toxicity. In particular, an Ig derived protein, specified fragment or variant of the invention, where the individual components, such as the variable region, constant region and framework, individually and/or collectively, optionally and preferably possess low immunogenicity, is useful in the present invention. The Ig derived proteins that can be used in the invention are optionally characterized by their ability to treat patients for extended periods with good to excellent alleviation of symptoms and low toxicity. Low immunogenicity and/or high affinity, as well as other suitable properties, may contribute to the therapeutic results achieved. "Low immunogenicity" is defined herein as raising significant HAHA, HACA or HAMA responses in less than about 75%, or preferably less than about 50% of the patients treated and/or raising low titres in the patient treated (less than about 300, preferably less than about 100 measured with a double antigen enzyme immunoassay) (Elliott *et al.*, *Lancet* 344:1125-1127 (1994), entirely incorporated herein by reference).

The invention includes an isolated nucleic acid molecule that encodes RELP protein. The molecule can be a nucleic acid molecule of Seq ID No 1, a nucleic acid molecule encoding a protein having at least a 70% identity to a polypeptide comprising amino acids of SEQ ID NO:2.

The invention also encompasses a nucleic acid molecule that is complementary to the molecule that encodes a protein having at least 70% identity to Seq. ID No. 2, a nucleic acid

molecule of at least 15 sequential bases of the nucleic acid sequence of Seq. ID No. 1, or a nucleic acid molecule that hybridizes under stringent conditions to the nucleic acid sequence molecule of Seq. ID No. 1.

In another aspect of the invention, isolated RELP is presented.

5 In yet another aspect of the invention methods of detecting the presence of a tumor or a cancerous condition includes detecting the expression of polypeptides, proteins, or nucleic acid molecules having the sequences described above and correlating the presence or concentration of such molecule in a biological sample with the presence or absence of said tumor or cancerous event.

10 In yet another aspect of the invention, antibodies that binds to the RELP and functional equivalents thereof are presented.

In yet another aspect of the invention, kits for detecting the polypeptides, proteins, or nucleic acid sequences described above are presented.

Utility

15 The isolated nucleic acids of the present invention can be used for production of at least one RELP Ig derived protein, fragment or specified variant thereof, which can be used to effect in a cell, tissue, organ or animal (including mammals and humans), to modulate, treat, alleviate, help prevent the incidence of, or reduce the symptoms of, at least one RELP protein condition, selected from, but not limited to, at least one malignant disorder or disease, as well as other known or specified RELP protein related conditions.

20 Such a method can comprise administering an effective amount of a composition or a pharmaceutical composition comprising at least one RELP Ig derived protein or specified portion or variant to a cell, tissue, organ, animal or patient in need of such modulation, treatment, alleviation, prevention, or reduction in symptoms, effects or mechanisms. The 25 effective amount can comprise an amount of about 0.001 to 500 mg/kg per single or multiple administration, or to achieve a serum concentration of 0.01-5000 µg/ml serum concentration per single or multiple administration, or any effective range or value therein, as done and determined using known methods, as described herein or known in the relevant arts.

Citations

30 All publications or patents cited herein are entirely incorporated herein by reference as they show the state of the art at the time of the present invention and/or to provide description and enablement of the present invention. Publications refer to any scientific or patent publications, or any other information available in any media format, including all recorded,

electronic or printed formats. The following references are entirely incorporated herein by reference: Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, NY (1987-2001); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, NY (1989); Harlow and Lane, Ig derived proteins, a Laboratory Manual, Cold Spring Harbor, NY (1989); Colligan, et al., eds., Current Protocols in Immunology, John Wiley & Sons, Inc., NY (1994-2001); Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, NY, (1997-2001).

10 **RELP Proteins and methods of making and using.**

Definitions:

The term "protein superfamily" as used herein refers to proteins whose evolutionary relationship may not be entirely established or may be distant by accepted phylogenetic standards, but show similar three dimensional structure or display unique consensus of critical amino acids. The term "protein family" as used herein refers to proteins whose evolutionary relationship has been established by accepted phylogenetic standards.

As used herein, the term nucleic acid sequence includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "nucleic acid sequences" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are nucleic acid sequences as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term nucleic acid sequence as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acid sequences, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, *inter alia*. Nucleic acid sequences embraces short nucleic acid sequences often referred to as oligonucleotide(s).

As used herein, a "functional derivative" of RELP is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of RELP. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of RELP. A molecule is "substantially similar" to RELP if both molecules have substantially similar structures or if both molecules possess similar biological activity.

A newly identified protein, "RELP" (Reg Like Protein), is characterized in this specification. Nucleic acids (including, for example, cDNA) encoding for this protein have been isolated and cloned and uses for this protein in cancer diagnostics are presented. The gene structure and its chromosomal location are presented, and the tissue distribution of its expression is described. Additionally, antibodies that bind to this protein have been prepared and methods for their use have been devised. The murine homologue of RELP was also cloned and characterized.

All nucleic acid sequences described in this specification are shown in the 5'→3' direction unless otherwise indicated.

Fig. 1 shows the nucleic acid sequence of a cDNA (Seq. ID No. 1) used to produce RELP. The RELP cDNA encodes a 158-amino acid protein with a putative 22-amino acid signal peptide (Fig. 3). The molecular weight of RELP is about 18kd, and the isoelectric point was calculated as 9.128. The aminotermminus of RELP is highly hydrophobic and contains a cleavable signal sequence of 22 aminoacids. Human Reg proteins are 51-87% identical and 55-87% similar to each other, whereas RELP is 32-37% identical and 42-47% similar to them.

The primary structure of RELP is similar to that of the subgroup of C-type lectin superfamily of proteins, which contain a single carbohydrate-recognition (CRD) domain. The CRD-associated four conserved and two optional cysteines involved in intramolecular disulphide bonds are all conserved in RELP. Residues 50-53 represent a putative N-glycosylation site. The secondary structure of RELP is similar to that of human Reg1 α and the global folds of these proteins appear to be related. The amino acid sequence of RELP is shown in Fig. 2 (Seq. ID No. 2).

The RELP gene resides on chromosome 1 band p12-13.1 and spans about 17,500 base pairs. It is comprised of seven exons. Fig. 4 shows a schematic representation of the gene with the distance between exons scaled. The location of each exon is shown in Roman numerals.

Expression of RELP in normal tissues: RELP message is highly expressed in a subset of epithelial cells in the small intestine. This subset of cells represents the intestinal neuroendocrine cells (verified by colocalization of chromogranin). RELP mRNA is also seen in the stomach, various parts of the colon, where it is localized in the epithelial cells in the crypt bottom, the pancreas, the prostate and the testis.

Expression of RELP in diseased tissues: RELP is ectopically abundantly expressed in mucinous tumors originating from various organs, such as ovary, stomach, colon, breast and pancreas. The expression of RELP mRNA appears to be extremely high in mucinous ovarian

tumors. On a protein level a high, uniform expression is seen in the epithelial cells from mucinous ovarian, stomach, colon and breast tumors. Intraductal mucinous pancreatic tumors also express RELP. These tumors are emerging as a newly identified entity of pancreatic disorders that predispose recurrent pancreatitis. They are probably apt to become malignant.

5 Biological samples from a subject are used to determine whether cancer cells are present in the subject. Examples of suitable samples include blood and biopsy material. One method of diagnosis is to expose RNA from cells in the sample to a labeled probe that is capable of hybridizing to the RELP gene transcript, or a fragment thereof, under stringent conditions. Of course, the hybridizing conditions are altered to achieve optimum sensitivity
10 and specificity depending on the nature of the biological sample, type of cancer, method of probe preparation, and method of tissue preparation.

15 After contacting the sample with the probe, the next step is determining whether the probe has hybridized with nucleotide sequences of the mRNA from the sample, from which the expression of the RELP gene is inferred, the presence at elevated levels being diagnostic of cancer.

20 Another diagnostic method is to contact a sample with antibodies directed to antigenic (i.e. RELP) peptides. These antibodies are useful in the development of very specific assays for the detection of RELP antigen, and allow the tests to be carried out in many different formats. Preferably, the antibodies are labeled monoclonal antibodies. Since RELP is a secreted molecule, detecting RELP antigen in body fluids, such as serum, plasma, cyst fluids, pancreatic juice, and urine can be used to detect or follow-up RELP-expressing cancers. Typically, the protein is expressed between 100 and 1000 times in diseased tissues (as described above) compared with its normal expression levels. Accordingly, serum levels of 200 to 1000 % those of normal levels will be detected in the serum assays of this invention.
25 Most typically, a serum level of about 250% that of normal RELP levels can be expected in patients with colon cancer. Likewise, in molecular diagnostic tests in which mRNA expression levels are assayed, expression levels that are 150 to 1000 % those of normal levels indicate disease.

30 Purified biologically active RELP may have several different physical forms. RELP may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent RELP polypeptide may be posttranslationally modified by specific proteolytic cleavage events that results in the formation of fragments of the full length nascent polypeptide. A fragment, or

physical association of fragments may have the full biological activity associated with RELP however, the degree of RELP activity may vary between individual RELP fragments and physically associated RELP polypeptide fragments.

Since there is a substantial amount of redundancy in the various codons that code for specific amino acids, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein, which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of aliphatic amino acids alanine, valine, leucine and isoleucine; interchange of the hydroxyl residues serine and threonine, exchange of the acidic residues aspartic acid and glutamic acid, substitution between the amide residues asparagine and glutamine, exchange of the basic residues lysine and arginine and among the aromatic residues phenylalanine, tyrosine may not cause a change in functionality of the polypeptide. Such substitutions are well known and are described, for instance in Molecular Biology of the Gene, 4th Ed. Bengamin Cummings Pub. Co. by Watson et al.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis, chimeric substitution, and gene fusions. Site-directed mutagenesis is used to change one or more DNA residues that may result in a silent mutation, a conservative mutation, or a nonconservative mutation. Chimeric genes are prepared by swapping domains of similar or different genes to replace similar domains in the RELP gene. Similarly, fusion genes may be prepared that add domains to the RELP gene, such as an affinity tag to facilitate identification and isolation of the gene. Fusion genes may be prepared to replace regions of the RELP gene, for example to create a soluble version of the protein by removing a transmembrane domain or adding a targeting sequence to redirect the normal transport of the protein, or adding new post-translational modification sequences to the RELP gene. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand. All such changes of the nucleic acid sequence or polypeptide sequences are anticipated as useful variants of the present invention so long as they retain their functionality consistent with the original use of the nucleic acid sequence or polypeptide sequence of the present invention as described herein.

Identity or similarity, as known in the art, are relationships between two or more polypeptide sequences or two or more nucleic acid sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or nucleic acid sequence sequences, as the case may be, as determined by the 5 match between strings of such sequences. Both identity and similarity can be readily calculated (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular 10 Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity and similarity between two nucleic acid sequences or two polypeptide sequences, both terms are well known to skilled artisans (Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, 15 Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., (1988) SIAM J. Applied Math., 48, 1073. Methods commonly employed to determine identity or similarity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., (1988) SIAM J. Applied Math., 48, 1073. Preferred methods to determine identity are designed to give the largest match between the sequences 20 tested. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., (1984) Nucleic Acids Research 12(1), 387), BLASTP, BLASTN, and FASTA (Atschul, S. F. et al., (1990) J. Molec. Biol. 215, 403).

25 Polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids. Many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that 30 occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present are, to name an illustrative few, acetylation, acylation, ADP- ribosylation, amidation, covalent attachment of flavin, covalent

attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-
5 carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill and have been described in great detail in the scientific literature. c.f. PROTEINS-- STRUCTURE
10 AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993).

Included within the scope of the invention are nucleic acid sequences that are at least 70% identical over their entire length to a nucleic acid sequence encoding the polypeptide having the amino acid sequences set out herein, and nucleic acid sequences which are
15 complementary to such nucleic acid sequences. Alternatively, highly preferred are nucleic acid sequences that comprise a region that is at least 80% identical, more highly preferred are nucleic acid sequences that comprise a region that is at least 90% identical, and among these preferred nucleic acid sequences, those with at least 95% are especially preferred.
Furthermore, those with at least 97% identity are highly preferred among those with at least
20 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the most preferred. The nucleic acid sequences which hybridize to the hereinabove described nucleic acid sequences in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the polypeptide characterized by the RELP amino acid sequences set forth herein. Preferred
25 embodiments in this respect, moreover, are nucleic acid sequences that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptide encoded by the DNA of Seq. Id No. 1. The present invention further relates to nucleic acid sequences that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to nucleic acid sequences that hybridize under stringent conditions to the herein above-described nucleic acid sequences. As herein used, the term "stringent
30 conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

Nucleic acid sequences of the invention may be used as a hybridization probe for RNA, cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding the

sequences of RELP set forth herein and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to them. Such probes generally will comprise at least 15 bases. Preferably, such probes will have at least 30 bases and may have at least 50 bases. Particularly preferred probes will have at least 30 bases and will have 50 bases or less. For example, the coding region of the gene of the invention may be isolated by screening using the known DNA sequence to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the present invention is then used to screen a library of cDNA, genomic DNA or mRNA to determine to which members of the library the probe hybridizes.

The polypeptides of the present invention include the polypeptide of Seq. ID No. 2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% identity to the polypeptide of Seq. ID No. 2, preferably at least 80% identity to the polypeptide of Seq. ID No. 2, and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of Seq. ID No. 2 and still more preferably at least 95% similarity (still more preferably at least 97% identity) to the polypeptide of Seq. ID No. 2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids. Representative examples of polypeptide fragments of the invention, include, for example, truncation polypeptides of Seq. ID No. 2 or of variants or derivatives thereof, except for deletion of a continuous series of residues (that is, a continuous region, part or portion) that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or, as in double truncation mutants, deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Also preferred in this aspect of the invention are fragments characterized by structural or functional attributes of the polypeptide characterized by the sequences of Seq. ID No. 2.

Ig derived proteins of the Present Invention

The term "Ig derived protein" is intended to encompass Ig derived proteins, digestion fragments, specified portions and variants thereof, including Ig derived protein mimetics or comprising portions of Ig derived proteins that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain Ig derived proteins and fragments thereof. Functional fragments include antigen-binding fragments that bind to human RELP protein. For example, Ig derived protein fragments capable of binding to human RELP protein or portions thereof, including, but not limited to Fab (e.g., by papain digestion), Fab' (e.g., by pepsin digestion and partial reduction) and F(ab')₂ (e.g., by pepsin digestion),

factb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular biology techniques) fragments, are encompassed by the invention (see, e.g., Colligan, Immunology, supra).

Such fragments can be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Ig derived proteins can also be produced in a variety of truncated forms using Ig derived protein genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and/or hinge region of the heavy chain. The various portions of Ig derived proteins can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, a nucleic acid encoding the variable and constant regions of a human Ig derived protein chain can be expressed to produce a contiguous protein. See, e.g., Colligan, Immunology, supra, sections 2.8 and 2.10, for fragmentation and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*, 242: 423-426 (1988), regarding single chain Ig derived proteins, each of which publications are entirely incorporated herein by reference.

As used herein, the term "human Ig derived protein" refers to an Ig derived protein in which substantially every part of the protein (e.g., CDR, framework, C_L, C_H domains (e.g., C_H1, C_H2, C_H3), hinge, (V_L, V_H)) is substantially non-immunogenic, with only minor sequence changes or variations. Such changes or variations optionally and preferably retain or reduce the immunogenicity in humans relative to non-modified human Ig derived proteins. Thus, a human Ig derived protein is distinct from a chimeric or humanized Ig derived protein. It is pointed out that a human Ig derived protein can be produced by a non-human animal or prokaryotic or eukaryotic cell that is capable of expressing functionally rearranged human immunoglobulin (e.g., heavy chain and/or light chain) genes. Further, when a human Ig derived protein is a single chain Ig derived protein, it can comprise a linker peptide that is not found in native human Ig derived proteins. For example, an Fv can comprise a linker peptide, such as two to about eight glycine or other amino acid residues, which connects the variable region of the heavy chain and the variable region of the light chain. Such linker peptides are considered to be of human origin.

Human Ig derived proteins that are specific for human RELP proteins or fragments thereof can be raised against an appropriate immunogenic antigen, such as isolated and/or RELP protein or a portion thereof (including synthetic molecules, such as synthetic peptides).

Preparation of immunogenic antigens, and monoclonal Ig derived protein production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. 5 and D. Lane, 1988, *Ig derived proteins: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (e.g., Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991-2000)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, 10 NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U937, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, or the like, or heteromylomas, fusion products thereof, or any cell or fusion cell derived therefrom, or any other suitable cell line as known in the art, see, e.g., www.atcc.org, www.lifetech.com, and the like) with Ig derived protein 15 producing cells, such as, but not limited to, isolated or cloned spleen cells, or any other cells expressing heavy or light chain constant or variable or framework or CDR sequences, either as endogenous or heterologous nucleic acid, as recombinant or endogenous, viral, bacterial, algal, prokaryotic, amphibian, insect, reptilian, fish, mammalian, rodent, equine, ovine, goat, sheep, primate, eukaryotic, genomic DNA, cDNA, rDNA, mitochondrial DNA or RNA, chloroplast 20 DNA or RNA, hnRNA, mRNA, tRNA, single, double or triple stranded, hybridized, and the like or any combination thereof. See, e.g., Ausubel, supra, and Colligan, *Immunology*, supra, chapter 2, entirely incorporated herein by reference.

Ig derived protein producing cells can be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been 25 immunized with the antigen of interest. Any other suitable host cell can also be used for expressing heterologous or endogenous nucleic acid encoding an Ig derived protein, specified fragment or variant thereof, of the present invention. The fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods. Cells which 30 produce Ig derived proteins with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating Ig derived proteins of the requisite specificity can be used, including, but not limited to, methods that select recombinant Ig derived protein from a peptide or protein library (e.g., but not limited to, a bacteriophage or

ribosome display library; e.g., as available from Cambridge Ig derived protein Technologies, Cambridgeshire, UK; MorphoSys, Martinsreid/Planegg, DE; Biovation, Aberdeen, Scotland, UK; BioInvent, Lund, Sweden; Dyax Corp., Enzon, Affymax/Biosite; Xoma, Berkeley, CA; Ixsys; US pat. Nos. EP 368,684, PCT/GB91/01134; PCT/GB92/01755; PCT/GB92/002240;

5 PCT/GB92/00883; PCT/GB93/00605; US 08/350260(5/12/94); PCT/GB94/01422;
PCT/GB94/02662; PCT/GB97/01835; (CAT/MRC); WO90/14443; WO90/14424;
WO90/14430; PCT/US94/1234; WO92/18619; WO96/07754; (Scripps); EP 614 989
(MorphoSys); WO95/16027 (BioInvent); WO88/06630; WO90/3809 (Dyax); US 4,704,692
(Enzon); PCT/US91/02989 (Affymax); WO89/06283; EP 371 998; EP 550 400; (Xoma); EP
10 229 046; PCT/US91/07149 (Ixsys); or stochastically generated peptides or proteins - US
5723323, 5763192, 5814476, 5817483, 5824514, 5976862, WO 86/05803, EP 590 689 (Ixsys,
now Applied Molecular Evolution (AME), each entirely incorporated herein by reference) or
that rely upon immunization of transgenic animals (e.g., SCID mice, Nguyen et al., Microbiol.
Immunol. 41:901-907 (1997); Sandhu et al., Crit. Rev. Biotechnol. 16:95-118 (1996); Eren et
15 al., Immunol. 93:154-161 (1998), each entirely incorporated by reference as well as related
patents and application) that are capable of producing a repertoire of human Ig derived
proteins, as known in the art and/or as described herein. Additional techniques, include, but
are not limited to, ribosome display (Hanes et al., Proc. Natl. Acad. Sci. USA, 94:4937-4942
(May 1997); Hanes et al., Proc. Natl. Acad. Sci. USA, 95:14130-14135 (Nov. 1998)); single
20 cell Ig derived protein producing technologies (e.g., selected lymphocyte Ig derived protein
method ("SLAM") (US pat. No. 5,627,052, Wen et al., J. Immunol. 17:887-892 (1987);
Babcock et al., Proc. Natl. Acad. Sci. USA 93:7843-7848 (1996)); gel microdroplet and flow
cytometry (Powell et al., Biotechnol. 8:333-337 (1990); One Cell Systems, Cambridge, MA;
Gray et al., J. Imm. Meth. 182:155-163 (1995); Kenny et al., Bio/Technol. 13:787-790 (1995));
25 B-cell selection (Steenbakkers et al., Molec. Biol. Reports 19:125-134 (1994); Jonak et al.,
Progress Biotech, Vol. 5, In Vitro Immunization in Hybridoma Technology, Borrebaeck, ed.,
Elsevier Science Publishers B.V., Amsterdam, Netherlands (1988)).

Methods for humanizing non-human Ig derived proteins can also be used and are well
known in the art. Generally, a humanized antibody has one or more amino acid residues
30 introduced into it from a source which is non-human. These non-human amino acid residues
are often referred to as "import" residues, which are typically taken from an "import" variable
domain. Humanization can be essentially performed following the method of Winter and co-
workers (Jones et al., Nature 321:522 (1986); Riechmann et al., Nature 332:323 (1988);
Verhoeyen et al., Science 239:1534 (1988)), by substituting rodent CDRs or CDR sequences

for the corresponding sequences of a human antibody. Accordingly, such "humanized" Ig derived proteins are chimeric Ig derived proteins (Cabilly et al., *supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized Ig derived proteins are typically human Ig derived proteins in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent Ig derived proteins.

The choice of human variable domains, both light and heavy, to be used in making the humanized Ig derived proteins can be used to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.* 151: 2296 (1993); Chothia and Lesk, *J. Mol. Biol.* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human Ig derived proteins of a particular subgroup of light or heavy chains. The same framework can be used for several different humanized Ig derived proteins (Carter et al., *Proc. Natl. Acad. Sci. U.S.A.* 89:4285 (1992); Presta et al., *J. Immunol.* 151:2623 (1993)).

Ig derived proteins can also optionally be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized Ig derived proteins are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Human monoclonal Ig derived proteins can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal Ig derived proteins have been described, for example, by Kozbor, *J. Immunol.* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and*

Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner et al., J. Immunol. 147:86 (1991).

Alternatively, phage display technology (McCafferty et al., Nature 348:552 (1990)) and as presented above can be used to produce human Ig derived proteins and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson et al., Current Opinion in Structural Biology 3:564 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature 352:624 (1991) isolated a diverse array of anti-oxazolone Ig derived proteins from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and Ig derived proteins to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222:581 (1991), or Griffith et al., EMBO J. 12:725 (1993).

In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks et al., Bio/Technol. 10:779 (1992)). In this method, the affinity of "primary" human Ig derived proteins obtained by phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of Ig derived proteins and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by Waterhouse et al., Nucl. Acids Res. 21:2265 (1993). Gene shuffling can also be used to derive human Ig derived proteins from rodent Ig derived proteins, where the human antibody has similar affinities and specificities to the starting rodent antibody. According to this method, which is also referred to as "epitope imprinting", the

heavy or light chain V domain gene of rodent Ig derived proteins obtained by phage display technique is replaced with a repertoire of human V domain genes, creating rodent-human chimeras. Selection with antigen results in isolation of human variable capable of restoring a functional antigen-binding site, i.e. the epitope governs (imprints) the choice of partner. When
5 the process is repeated in order to replace the remaining rodent V domain, a human antibody is obtained (see PCT WO 93/06213, published 1 April 1993). Unlike traditional humanization of rodent Ig derived proteins by CDR grafting, this technique provides completely human Ig derived proteins, which have no framework or CDR residues of rodent origin.

Bispecific Ig derived proteins can also be used that are monoclonal, preferably human
10 or humanized, Ig derived proteins that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for at least one RELP protein, the other one is for any other antigen. For example, bispecific Ig derived proteins specifically binding a RELP protein and at least one neurotrophic factor, or two different types of RELP protein polypeptides are within the scope of the present invention.
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Methods for making bispecific Ig derived proteins are known in the art. Traditionally, the recombinant production of bispecific Ig derived proteins is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature 305:537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published 13 May 1993, and in Traunecker et al.,
20 EMBO J. 10:3655 (1991), entirely incorporated herein by reference.
25

According to a different and more preferred approach, antibody-variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant-domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, the second heavy chain constant region (C.sub.H 2), and the third heavy chain constant region (C.sub.H 3). It is
30 preferred to have the first heavy-chain constant region (C.sub.H 1), containing the site necessary for light-chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide

fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the production of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of
5 no particular significance. In a preferred embodiment of this approach, the bispecific Ig derived proteins are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain
10 combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. For further details of generating bispecific Ig derived proteins, see, for example, Suresh et al., Methods in Enzymology 121:210 (1986).

Heteroconjugate Ig derived proteins are also within the scope of the present invention.
15 Heteroconjugate Ig derived proteins are composed of two covalently joined Ig derived proteins. Such Ig derived proteins have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360; WO 92/00373; and EP 03089). Heteroconjugate Ig derived proteins can be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in
20 the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

In a preferred embodiment, at least one anti-RELP Ig derived protein or specified portion or variant of the present invention is produced by a cell line, a mixed cell line, an immortalized cell or clonal population of immortalized cells. Immortalized RELP protein
25 producing cells can be produced using suitable methods, for example, fusion of a human Ig derived protein-producing cell and a heteromyeloma or immortalization of an activated human B cell via infection with Epstein Barr virus (Niedbala et al., *Hybridoma*, 17(3):299-304 (1998); Zanella et al., *J Immunol Methods*, 156(2):205-215 (1992); Gustafsson et al., *Hum Ig derived proteins Hybridomas*, 2(1):26-32 (1991)). Preferably, the human anti-human RELP Ig derived
30 protein or specified portion or variant is generated by immunization of a transgenic animal (e.g., mouse, rat, hamster, non-human primate, and the like) capable of producing a repertoire of human Ig derived proteins, as described herein and/or as known in the art. Cells that produce a human anti-human RELP Ig derived protein can be isolated from such animals and immortalized using suitable methods, such as the methods described herein.

Transgenic mice that can produce a repertoire of human Ig derived proteins that bind to human antigens can be produced by known methods (e.g., but not limited to, U.S. Pat. Nos: 5,770,428, 5,569,825, 5,545,806, 5,625,126, 5,625,825, 5,633,425, 5,661,016 and 5,789,650 issued to Lonberg *et al.*; Jakobovits *et al.* WO 98/50433, Jakobovits *et al.* WO 98/24893,
5 Lonberg *et al.* WO 98/24884, Lonberg *et al.* WO 97/13852, Lonberg *et al.* WO 94/25585, Kucherlapate *et al.* WO 96/34096, Kucherlapate *et al.* EP 0463 151 B1, Kucherlapate *et al.* EP 0710 719 A1, Surani *et al.* US. Pat. No. 5,545,807, Bruggemann *et al.* WO 90/04036, Bruggemann *et al.* EP 0438 474 B1, Lonberg *et al.* EP 0814 259 A2, Lonberg *et al.* GB 2 272 440 A, Lonberg *et al.* *Nature* 368:856-859 (1994), Taylor *et al.*, *Int. Immunol.* 6(4)579-591
10 (1994), Green *et al.*, *Nature Genetics* 7:13-21 (1994), Mendez *et al.*, *Nature Genetics* 15:146-156 (1997), Taylor *et al.*, *Nucleic Acids Research* 20(23):6287-6295 (1992), Tuailon *et al.*, *Proc Natl Acad Sci USA* 90(8)3720-3724 (1993), Lonberg *et al.*, *Int Rev Immunol* 13(1):65-93 (1995) and Fishwald *et al.*, *Nat Biotechnol* 14(7):845-851 (1996), which are each entirely incorporated herein by reference). Generally, these mice comprise at least one transgene comprising DNA from at least one human immunoglobulin locus that is functionally rearranged, or which can undergo functional rearrangement. The endogenous immunoglobulin loci in such mice can be disrupted or deleted to eliminate the capacity of the animal to produce Ig derived proteins encoded by endogenous genes.
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The term “functionally rearranged,” as used herein refers to a segment of DNA from an immunoglobulin locus that has undergone V(D)J recombination, thereby producing an immunoglobulin gene that encodes an immunoglobulin chain (e.g., heavy chain, light chain), or any portion thereof. A functionally rearranged immunoglobulin gene can be directly or indirectly identified using suitable methods, such as, for example, nucleotide sequencing, hybridization (e.g., Southern blotting, Northern blotting) using probes that can anneal to coding joints between gene segments or enzymatic amplification of immunoglobulin genes (e.g., polymerase chain reaction) with primers that can anneal to coding joints between gene segments. Whether a cell produces an Ig derived protein comprising a particular variable region or a variable region comprising a particular sequence (e.g., at least one CDR sequence) can also be determined using suitable methods. In one example, mRNA can be isolated from an Ig derived protein-producing cell (e.g., a hybridoma or recombinant cell or other suitable source) and used to produce cDNA encoding the Ig derived protein or specified portion or variant thereof. The cDNA can be cloned and sequenced or can be amplified (e.g., by polymerase chain reaction or other known and suitable methods) using a first primer that
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anneals specifically to a portion of the variable region of interest (e.g., CDR, coding joint) and a second primer that anneals specifically to non-variable region sequences (e.g., C_H1, V_H).

Screening Ig derived protein or specified portion or variants for specific binding to similar proteins or fragments can be conveniently achieved using peptide display libraries. This method involves the screening of large collections of peptides for individual members having the desired function or structure. Ig derived protein screening of peptide display libraries is well known in the art. The displayed peptide sequences can be from 3 to 5000 or more amino acids in length, frequently from 5-100 amino acids long, and often from about 8 to 25 amino acids long.

In addition to direct chemical synthetic methods for generating peptide libraries, several recombinant DNA methods have been described. One type involves the display of a peptide sequence on the surface of a bacteriophage or cell. Each bacteriophage or cell contains the nucleotide sequence encoding the particular displayed peptide sequence. Such methods are described in PCT Patent Publication Nos. 91/17271, 91/18980, 91/19818, and 93/08278. Other systems for generating libraries of peptides have aspects of both in vitro chemical synthesis and recombinant methods. See, PCT Patent Publication Nos. 92/05258, 92/14843, and 96/19256.

See also, U.S. Patent Nos. 5,658,754; and 5,643,768. Peptide display libraries, vector, and screening kits are commercially available from such suppliers as Invitrogen (Carlsbad, CA), and Cambridge Ig derived protein Technologies (Cambridgeshire, UK). See, e.g., U.S. Pat. Nos. 4704692, 4939666, 4946778, 5260203, 5455030, 5518889, 5534621, 5656730, 5763733, 5767260, 5856456, assigned to Enzon; 5223409, 5403484, 5571698, 5837500, assigned to Dyax, 5427908, 5580717, assigned to Affymax; 5885793, assigned to Cambridge Ig derived protein Technologies; 5750373, assigned to Genentech, 5618920, 5595898, 5576195, 5698435, 5693493, 5698417, assigned to Xoma, Colligan, *supra*; Ausubel, *supra*; or Sambrook, *supra*, each of the above patents and publications entirely incorporated herein by reference.

Ig derived proteins, specified portions and variants of the present invention can also be prepared using at least one RELP Ig derived protein or specified portion or variant encoding nucleic acid to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such Ig derived proteins or specified portions or variants in their milk. Such animals can be provided using known methods. See, e.g., but not limited to, US patent nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; 5,304,489, and the like, each of which is entirely incorporated herein by reference.

Ig derived proteins, specified portions and variants of the present invention can additionally be prepared using at least one RELP Ig derived protein or specified portion or variant encoding nucleic acid to provide transgenic plants and cultured plant cells (e.g., but not

limited to tobacco and maize) that produce such Ig derived proteins, specified portions or variants in the plant parts or in cells cultured therefrom. As a non-limiting example, transgenic tobacco leaves expressing recombinant proteins have been successfully used to provide large amounts of recombinant proteins, e.g., using an inducible promoter. See, e.g., Cramer et al.,
5 Curr. Top. Microbiol. Immunol. 240:95-118 (1999) and references cited therein. Also, transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood et al., Adv. Exp. Med. Biol. 464:127-147 (1999)
and references cited therein. Ig derived proteins have also been produced in large amounts
10 from transgenic plant seeds including Ig derived protein fragments, such as single chain Ig derived proteins (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad et al., Plant Mol. Biol. 38:101-109 (1998) and reference cited therein. Thus, Ig derived proteins, specified portions and variants of the present invention can also be produced using transgenic plants, according to known methods. See also, e.g., Fischer et al., Biotechnol. Appl. Biochem.
15 30:99-108 (Oct., 1999), Ma et al., Trends Biotechnol. 13:522-7 (1995); Ma et al., Plant Physiol. 109:341-6 (1995); Whitelam et al., Biochem. Soc. Trans. 22:940-944 (1994); and references cited therein. See, also generally for plant expression of Ig derived proteins, but not limited to, Each of the above references is entirely incorporated herein by reference.

The Ig derived proteins of the invention can bind human RELP protein with a wide range of affinities (K_D). In a preferred embodiment, at least one human mAb of the present invention can optionally bind human RELP protein with high affinity. For example, a human mAb can bind human RELP protein with a K_D equal to or less than about 10^{-9} M or, more preferably, with a K_D equal to or less than about 0.1-9.9 (or any range or value therein) $\times 10^{-10}$ M, 10^{-11} , 10^{-12} , 10^{-13} or any range or value therein.
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The affinity or avidity of an Ig derived protein for an antigen can be determined experimentally using any suitable method. (See, for example, Berzofsky, *et al.*, "Ig derived protein-Antigen Interactions," In *Fundamental Immunology*, Paul, W. E., Ed., Raven Press: New York, NY (1984); Kuby, Janis *Immunology*, W. H. Freeman and Company: New York, NY (1992); and methods described herein). The measured affinity of a particular Ig derived protein-antigen interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of affinity and other antigen-binding parameters (e.g., K_D , K_a , K_d) are preferably made with standardized solutions of Ig derived protein and antigen, and a standardized buffer, such as the buffer described herein.
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Nucleic Acid Molecules

Using the information provided herein, such as the nucleotide sequences encoding at least 90-100% of the contiguous amino acids of at least one of RELP Ig derived protein, specified fragments, variants or consensus sequences thereof, or a deposited vector comprising at least one of these sequences, a nucleic acid molecule of the present invention encoding at least one RELP Ig derived protein or specified portion or variant can be obtained using methods described herein or as known in the art.

Nucleic acid molecules of the present invention can be in the form of RNA, such as mRNA, hnRNA, tRNA or any other form, or in the form of DNA, including, but not limited to, cDNA and genomic DNA obtained by cloning or produced synthetically, or any combinations thereof. The DNA can be triple-stranded, double-stranded or single-stranded, or any combination thereof. Any portion of at least one strand of the DNA or RNA can be the coding strand, also known as the sense strand, or it can be the non-coding strand, also referred to as the anti-sense strand.

Isolated nucleic acid molecules of the present invention can include nucleic acid molecules comprising an open reading frame (ORF), optionally with one or more introns, e.g., but not limited to, at least one specified portion of at least one CDR, as CDR1, CDR2 and/or CDR3 of at least one heavy chain or light chain, respectively; nucleic acid molecules comprising the coding sequence for a RELP Ig derived protein or specified portion or variant; and nucleic acid molecules which comprise a nucleotide sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode at least one RELP Ig derived protein as described herein and/or as known in the art. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate nucleic acid variants that code for specific RELP Ig derived protein or specified portion or variants of the present invention. See, e.g., Ausubel, et al., *supra*, and such nucleic acid variants are included in the present invention.

In another aspect, the invention provides isolated nucleic acid molecules encoding a(n) RELP Ig derived protein or specified portion or variant having an amino acid sequence as encoded by the nucleic acid contained in the plasmid deposited as designated clone names _____ and ATCC Deposit Nos.

30 _____, respectively, deposited on _____.

As indicated herein, nucleic acid molecules of the present invention which comprise a nucleic acid encoding a RELP Ig derived protein or specified portion or variant can include, but are not limited to, those encoding the amino acid sequence of an Ig derived protein

fragment, by itself; the coding sequence for the entire Ig derived protein or a portion thereof; the coding sequence for an Ig derived protein, fragment or portion, as well as additional sequences, such as the coding sequence of at least one signal leader or fusion peptide, with or without the aforementioned additional coding sequences, such as at least one intron, together with additional, non-coding sequences, including but not limited to, non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals (for example - ribosome binding and stability of mRNA); an additional coding sequence that codes for additional amino acids, such as those that provide additional functionalities. Thus, the sequence encoding an Ig derived protein or specified portion or variant can be fused to a marker sequence, such as a sequence encoding a peptide that facilitates purification of the fused Ig derived protein or specified portion or variant comprising an Ig derived protein fragment or portion.

Polynucleotides Which Selectively Hybridize to a Polynucleotide as Described Herein

The present invention provides isolated nucleic acids that hybridize under selective hybridization conditions to a polynucleotide disclosed herein. Thus, the polynucleotides of this embodiment can be used for isolating, detecting, and/or quantifying nucleic acids comprising such polynucleotides. For example, polynucleotides of the present invention can be used to identify, isolate, or amplify partial or full-length clones in a deposited library. In some embodiments, the polynucleotides are genomic or cDNA sequences isolated, or otherwise complementary to, a cDNA from a human or mammalian nucleic acid library.

Preferably, the cDNA library comprises at least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low or moderate stringency hybridization conditions are typically, but not exclusively, employed with sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

Optionally, polynucleotides of this invention will encode at least a portion of an Ig derived protein or specified portion or variant encoded by the polynucleotides described herein. The polynucleotides of this invention embrace nucleic acid sequences that can be employed for selective hybridization to a polynucleotide encoding an Ig derived protein or specified portion or variant of the present invention. See, e.g., Ausubel, *supra*; Colligan, *supra*, each entirely incorporated herein by reference.

Construction of Nucleic Acids

The isolated nucleic acids of the present invention can be made using (a) recombinant methods, (b) synthetic techniques, (c) purification techniques, or combinations thereof, as well-known in the art.

The nucleic acids can conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites can be inserted into the nucleic acid to aid in isolation of the polynucleotide.

Also, translatable sequences can be inserted to aid in the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention. The nucleic acid of the present invention - excluding the coding sequence - is optionally a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention.

Additional sequences can be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art. (See, e.g., Ausubel, *supra*; or Sambrook, *supra*)

Recombinant Methods for Constructing Nucleic Acids

The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or any combination thereof, can be obtained from biological sources using any number of cloning methodologies known to those of skill in the art. In some embodiments, oligonucleotide probes that selectively hybridize, under stringent conditions, to the polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library. The isolation of RNA, and construction of cDNA and genomic libraries, is well known to those of ordinary skill in the art. (See, e.g., Ausubel, *supra*; or Sambrook, *supra*)

Nucleic Acid Screening and Isolation Methods

A cDNA or genomic library can be screened using a probe based upon the sequence of a polynucleotide of the present invention, such as those disclosed herein. Probes can be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different organisms. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The

degree of stringency can be controlled by one or more of temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through, for example, manipulation of the concentration of formamide within the range of 0% to 50%. The
5 degree of complementarity (sequence identity) required for detectable binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100%, or 90-100%, or any range or value therein. However, it should be understood that minor sequence variations in the probes and primers can be compensated for by reducing the stringency of the hybridization and/or wash medium.

10 Methods of amplification of RNA or DNA are well known in the art and can be used according to the present invention without undue experimentation, based on the teaching and guidance presented herein.

Known methods of DNA or RNA amplification include, but are not limited to, polymerase chain reaction (PCR) and related amplification processes (see, e.g., U.S. Patent
15 Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, to Mullis, et al.; 4,795,699 and 4,921,794 to Tabor, et al; 5,142,033 to Innis; 5,122,464 to Wilson, et al.; 5,091,310 to Innis; 5,066,584 to Gyllensten, et al; 4,889,818 to Gelfand, et al; 4,994,370 to Silver, et al; 4,766,067 to Biswas; 4,656,134 to Ringold) and RNA mediated amplification that uses anti-sense RNA to the target sequence as a template for double-stranded DNA synthesis (U.S. Patent No. 5,130,238 to
20 Malek, et al, with the tradename NASBA), the entire contents of which references are incorporated herein by reference. (See, e.g., Ausubel, *supra*; or Sambrook, *supra*.)

For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of polynucleotides of the present invention and related genes directly from genomic DNA or cDNA libraries. PCR and other in vitro amplification methods can also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. Examples of techniques sufficient to direct persons of skill through in vitro amplification methods are found in Berger, *supra*, Sambrook, *supra*, and Ausubel, *supra*, as well as Mullis, et al., U.S. Patent No. 4,683,202 (1987); and Innis, et al., PCR
30 Protocols A Guide to Methods and Applications, Eds., Academic Press Inc., San Diego, CA (1990). Commercially available kits for genomic PCR amplification are known in the art. See, e.g., Advantage-GC Genomic PCR Kit (Clontech). The T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by known methods (see, e.g., Ausubel, et al., *supra*). Chemical synthesis generally produces a single-stranded oligonucleotide, which can be converted into double-stranded DNA by hybridization with a complementary sequence, or by polymerization with a 5 DNA polymerase using the single strand as a template. One of skill in the art will recognize that while chemical synthesis of DNA can be limited to sequences of about 100 or more bases, longer sequences can be obtained by the ligation of shorter sequences.

Recombinant Expression Cassettes

The present invention further provides recombinant expression cassettes comprising a 10 nucleic acid of the present invention. A nucleic acid sequence of the present invention, for example a cDNA or a genomic sequence encoding an Ig derived protein or specified portion or variant of the present invention, can be used to construct a recombinant expression cassette that can be introduced into at least one desired host cell. A recombinant expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional 15 initiation regulatory sequences that will direct the transcription of the polynucleotide in the intended host cell. Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the nucleic acids of the present invention.

In some embodiments, isolated nucleic acids that serve as promoter, enhancer, or other elements can be introduced in the appropriate position (upstream, downstream or in intron) of a 20 non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example, endogenous promoters can be altered *in vivo* or *in vitro* by mutation, deletion and/or substitution.

A polynucleotide of the present invention can be expressed in either sense or anti-sense orientation as desired. It will be appreciated that control of gene expression in either sense or 25 anti-sense orientation can have a direct impact on the observable characteristics.

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes.

A variety of cross-linking agents, alkylating agents and radical generating species as 30 pendant groups on polynucleotides of the present invention can be used to bind, label, detect and/or cleave nucleic acids. Knorre, et al., Biochimie 67:785-789 (1985); Vlassov, et al., Nucleic Acids Res. 14:4065-4076 (1986); Iverson and Dervan, J. Am. Chem. Soc. 109:1241-1243 (1987); Meyer, et al., J. Am. Chem. Soc. 111:8517-8519 (1989); Lee, et al., Biochemistry 27:3197-3203 (1988); Home, et al., J. Am. Chem. Soc. 112:2435-2437 (1990); Webb and Matteucci, J. Am.

Chem. Soc. 108:2764-2765 (1986); Nucleic Acids Res. 14:7661-7674 (1986); Feteritz, et al., J. Am. Chem. Soc. 113:4000 (1991). Various compounds to bind, detect, label, and/or cleave nucleic acids are known in the art. See, for example, U.S. Patent Nos. 5,543,507; 5,672,593; 5,484,908; 5,256,648; and 5,681,941, each entirely incorporated herein by reference.

5

Vectors And Host Cells

The present invention also relates to vectors that include isolated nucleic acid molecules of the present invention, host cells that are genetically engineered with the recombinant vectors, and the production of at least one RELP Ig derived protein or specified portion or variant by recombinant techniques, as is well known in the art. See, e.g., Sambrook, et al., *supra*; Ausubel, et al., *supra*, each entirely incorporated herein by reference.

The polynucleotides can optionally be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it can be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

The DNA insert should be operatively linked to an appropriate promoter. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (e.g., UAA, UGA or UAG) appropriately positioned at the end of the mRNA to be translated, with UAA and UAG preferred for mammalian or eukaryotic cell expression.

Expression vectors will preferably but optionally include at least one selectable marker. Such markers include, e.g., but not limited to, methotrexate (MTX), dihydrofolate reductase (DHFR, US Pat.Nos. 4,399,216; 4,634,665; 4,656,134; 4,956,288; 5,149,636; 5,179,017, ampicillin, neomycin (G418), mycophenolic acid, or glutamine synthetase (GS, US Pat.Nos. 5,122,464; 5,770,359; 5,827,739) resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria or prokaryotics (the above patents are entirely incorporated hereby by reference). Appropriate culture mediums and conditions for the above-described host cells are known in the art. Suitable vectors will be readily apparent to the skilled artisan. Introduction of a vector construct into a host cell can be effected by calcium phosphate transfection, DEAE-dextran

mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other known methods. Such methods are described in the art, such as Sambrook, supra, Chapters 1-4 and 16-18; Ausubel, supra, Chapters 1, 9, 13, 15, 16.

At least one Ig derived protein or specified portion or variant of the present invention can be expressed in a modified form, such as a fusion protein, and can include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, can be added to the N-terminus of an Ig derived protein or specified portion or variant to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties can be added to an Ig derived protein or specified portion or variant of the present invention to facilitate purification. Such regions can be removed prior to final preparation of an Ig derived protein or at least one fragment thereof. Such methods are described in many standard laboratory manuals, such as Sambrook, supra, Chapters 17.29-17.42 and 18.1-18.74; Ausubel, supra, Chapters 16, 17 and 18.

Those of ordinary skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention.

Alternatively, nucleic acids of the present invention can be expressed in a host cell by turning on (by manipulation) in a host cell that contains endogenous DNA encoding an Ig derived protein or specified portion or variant of the present invention. Such methods are well known in the art, e.g., as described in US patent Nos. 5,580,734, 5,641,670, 5,733,746, and 5,733,761, entirely incorporated herein by reference.

Illustrative of cell cultures useful for the production of the Ig derived proteins, specified portions or variants thereof, are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions or bioreactors can also be used. A number of suitable host cell lines capable of expressing intact glycosylated proteins have been developed in the art, and include the COS-1 (e.g., ATCC CRL 1650), COS-7 (e.g., ATCC CRL-1651), HEK293, BHK21 (e.g., ATCC CRL-10), CHO (e.g., ATCC CRL 1610) and BSC-1 (e.g., ATCC CRL-26) cell lines, Cos-7 cells, CHO cells, hep G2 cells, P3X63Ag8.653, SP2/0-Ag14, 293 cells, HeLa cells and the like, which are readily available from, for example, American Type Culture Collection, Manassas, Va. Preferred host cells include cells of lymphoid origin such as myeloma and lymphoma cells. Particularly preferred host cells are P3X63Ag8.653 cells (ATCC Accession Number CRL-1580) and SP2/0-Ag14 cells (ATCC Accession Number CRL-1851). In a particularly preferred embodiment, the recombinant cell is a P3X63Ab8.653 or a SP2/0-Ag14 cell.

Expression vectors for these cells can include one or more of the following expression control sequences, such as, but not limited to an origin of replication; a promoter (e.g., late or early SV40 promoters, the CMV promoter (US Pat.Nos. 5,168,062; 5,385,839), an HSV tk promoter, a pgk (phosphoglycerate kinase) promoter, an EF-1 alpha promoter (US Pat.No. 5,266,491), at least one human immunoglobulin promoter; an enhancer, and/or processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. See, e.g., Ausubel et al., *supra*; Sambrook, et al., *supra*. Other cells useful for production of nucleic acids or proteins of the present invention are known and/or available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (www.atcc.org) or other known or commercial sources.

When eukaryotic host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript can also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, et al., *J. Virol.* 45:773-781 (1983)). Additionally, gene sequences to control replication in the host cell can be incorporated into the vector, as known in the art.

Purification of an Ig derived protein or Specified Portion or Variant Thereof

A RELP Ig derived protein or specified portion or variant can be recovered and purified from recombinant cell cultures by well-known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be employed for purification. See e.g., Colligan, *Current Protocols in Immunology*, or *Current Protocols in Protein Science*, John Wiley & Sons, NY, NY, (1997-2000), e.g., Chapters 1, 4, 6, 8, 9, 10, each entirely incorporated herein by reference.

Ig derived proteins or specified portions or variants of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the Ig derived protein or specified portion or variant of the present invention can be glycosylated or can be non-glycosylated, with glycosylated preferred. Such methods are described in many standard laboratory manuals, such as Sambrook, *supra*, Sections 17.37-

17.42; Ausubel, *supra*, Chapters 10, 12, 13, 16, 18 and 20, Colligan, *Protein Science, supra*, Chapters 12-14, all entirely incorporated herein by reference.

RELP Ig derived proteinS, FRAGMENTS AND/OR VARIANTS

The isolated Ig derived proteins of the present invention comprise an Ig derived protein or specified portion or variant encoded by any one of the polynucleotides of the present invention as discussed more fully herein, or any isolated or prepared Ig derived protein or specified portion or variant thereof.

Preferably, the human Ig derived protein or antigen-binding fragment binds human RELP protein and, thereby substantially neutralizes the biological activity of the protein. An

Ig derived protein, or specified portion or variant thereof, that partially or preferably substantially neutralizes at least one biological activity of at least one RELP protein or fragment can bind the protein or fragment and thereby inhibit activitys mediated through the binding of RELP protein to the RELP protein receptor or through other RELP protein-dependent or mediated mechanisms. As used herein, the term “neutralizing Ig derived protein”

refers to an Ig derived protein that can inhibit an RELP protein-dependent activity by about 20-120%, preferably by at least about 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% or more depending on the assay. The capacity of an RELP Ig derived protein or specified portion or variant to inhibit an RELP protein-dependent activity is preferably assessed by at least one suitable RELP Ig derived protein or protein assay, as described herein and/or as known in the art.

A human Ig derived protein or specified portion or variant of the invention can be of any class (IgG, IgA, IgM, IgE, IgD, etc.) or isotype and can comprise a kappa or lambda light chain. In one embodiment, the human Ig derived protein or specified portion or variant comprises an IgG heavy chain or defined fragment, for example, at least one of isotypes, IgG1, IgG2, IgG3 or IgG4. Ig derived proteins of this type can be prepared by employing a

transgenic mouse or other transgenic non-human mammal comprising at least one human light chain (e.g., IgG, IgA and IgM (e.g., $\gamma 1, \gamma 2, \gamma 3, \gamma 4$) transgenes as described herein and/or as known in the art. In another embodiment, the anti-human RELP protein human Ig derived protein or specified portion or variant thereof comprises an IgG1 heavy chain and a IgG1 light chain.

At least one Ig derived protein or specified portion or variant of the invention binds at least one specified epitope specific to at least one RELP protein, subunit, fragment, portion or any combination thereof. The at least one epitope can comprise at least one Ig derived protein binding region that comprises at least one portion of said protein, which epitope is preferably

comprised of at least one extracellular, soluble, hydrophilic, external or cytoplasmic portion of said protein. The at least one specified epitope can comprise any combination of at least one amino acid sequence of at least 1-3 amino acids to the entire specified portion of contiguous amino acids of the sequences selected from the group consisting of SEQ ID

5 NOS:2, 4, 5, 6, 7, 8, 9, 10, 11.

Generally, the human Ig derived protein or antigen-binding fragment of the present invention will comprise an antigen-binding region that comprises at least one human complementarity determining region (CDR1, CDR2 and CDR3) or variant of at least one heavy chain variable region and at least one human complementarity determining region
10 (CDR1, CDR2 and CDR3) or variant of at least one light chain variable region. As a non-limiting example, the Ig derived protein or antigen-binding portion or variant can comprise at least one of the heavy chain, and/or a light chain CDR3. In a particular embodiment, the Ig derived protein or antigen-binding fragment can have an antigen-binding region that comprises at least a portion of at least one heavy chain CDR (i.e., CDR1, CDR2 and/or CDR3) having the
15 amino acid sequence of the corresponding CDRs 1, 2 and/or 3. In another particular embodiment, the Ig derived protein or antigen-binding portion or variant can have an antigen-binding region that comprises at least a portion of at least one light chain CDR (i.e., CDR1, CDR2 and/or CDR3) having the amino acid sequence of the corresponding CDRs 1, 2 and/or 3. In a preferred embodiment the three heavy chain CDRs and the three light chain CDRs of
20 the antibody or antigen-binding fragment have the amino acid sequence of the corresponding CDR of at least one mAb, as described herein. Such Ig derived proteins can be prepared by chemically joining together the various portions (e.g., CDRs, framework) of the Ig derived protein using conventional techniques, by preparing and expressing a (i.e., one or more) nucleic acid molecule that encodes the Ig derived protein using conventional techniques of recombinant DNA technology or by using any other suitable method.
25

The anti-human RELP protein human Ig derived protein can comprise at least one of a heavy or light chain variable region having a defined amino acid sequence. For example, in a preferred embodiment, the human anti-human RELP Ig derived protein comprises at least one of at least one heavy chain variable region and/or at least one light chain variable region.

30 Human Ig derived proteins that bind to human RELP protein and that comprise a defined heavy or light chain variable region can be prepared using suitable methods, such as phage display (Katsume, Y., et al., *Int J Mol. Med.*, 1(5):863-868 (1998)) or methods that employ transgenic animals, as known in the art and/or as described herein. For example, a transgenic mouse, comprising a functionally rearranged human immunoglobulin heavy chain transgene

and a transgene comprising DNA from a human immunoglobulin light chain locus that can undergo functional rearrangement, can be immunized with human RELP protein or a fragment thereof to elicit the production of Ig derived proteins. If desired, the Ig derived protein producing cells can be isolated and hybridomas or other immortalized Ig derived protein-producing cells can be prepared as described herein and/or as known in the art. Alternatively, the Ig derived protein, specified portion or variant can be expressed using the encoding nucleic acid or portion thereof in a suitable host cell.

The invention also relates to Ig derived proteins, antigen-binding fragments, immunoglobulin chains and CDRs comprising amino acids in a sequence that is substantially the same as an amino acid sequence described herein. Preferably, such Ig derived proteins or antigen-binding fragments and Ig derived proteins comprising such chains or CDRs can bind human RELP protein with high affinity (e.g., K_D less than or equal to about 10^{-9} M). Amino acid sequences that are substantially the same as the sequences described herein include sequences comprising conservative amino acid substitutions, as well as amino acid deletions and/or insertions. A conservative amino acid substitution refers to the replacement of a first amino acid by a second amino acid that has chemical and/or physical properties (e.g., charge, structure, polarity, hydrophobicity/ hydrophilicity) that are similar to those of the first amino acid. Conservative substitutions include replacement of one amino acid by another within the following groups: lysine (K), arginine (R) and histidine (H); aspartate (D) and glutamate (E); asparagine (N), glutamine (Q), serine (S), threonine (T), tyrosine (Y), K, R, H, D and E; alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), tryptophan (W), methionine (M), cysteine (C) and glycine (G); F, W and Y; C, S and T.

Amino Acid Codes

The amino acids that make up RELP Ig derived proteins or specified portions or variants of the present invention are often abbreviated. The amino acid designations can be indicated by designating the amino acid by its single letter code, its three letter code, name, or three nucleotide codon(s) as is well understood in the art (see Alberts, B., et al., Molecular Biology of The Cell, Third Ed., Garland Publishing, Inc., New York, 1994):

SINGLE LETTER CODE	THREE LETTER CODE	NAME	THREE NUCLEOTIDE CODON(S)
A	Ala	Alanine	GCA, GCC, GCG, GCU
C	Cys	Cysteine	UGC, UGU
D	Asp	Aspartic acid	GAC, GAU
E	Glu	Glutamic acid	GAA, GAG
F	Phe	Phenylalanine	UUC, UUU

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G	Gly	Glycine	GGA, GGC, GGG, GGU
H	His	Histidine	CAC, CAU
I	Ile	Isoleucine	AUA, AUC, AUU
K	Lys	Lysine	AAA, AAG
L	Leu	Leucine	UUA, UUG, CUA, CUC, CUG, CUU
M	Met	Methionine	AUG
N	Asn	Asparagine	AAC, AAU
P	Pro	Proline	CCA, CCC, CCG, CCU
Q	Gln	Glutamine	CAA, CAG
R	Arg	Arginine	AGA, AGG, CGA, CGC, CGG, CGU
S	Ser	Serine	AGC, AGU, UCA, UCC, UCG, UCU
T	Thr	Threonine	ACA, ACC, ACG, ACU
V	Val	Valine	GUA, GUC, GUG, GUU
W	Trp	Tryptophan	UGG
Y	Tyr	Tyrosine	UAC, UAU

A RELP Ig derived protein or specified portion or variant of the present invention can include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation, as specified herein.

5 Of course, the number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of amino acid substitutions, insertions or deletions for any given RELP protein polypeptide will not be more than 40, 30, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, such as 1-30 or any range or value therein, as specified herein.

10 Amino acids in a RELP Ig derived protein or specified portion or variant of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (e.g., Ausubel, *supra*, Chapters 8, 15; Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are 15 then tested for biological activity, such as, but not limited to at least one RELP protein neutralizing activity. Sites that are critical for Ig derived protein or specified portion or variant binding can also be identified by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, et al., *J. Mol. Biol.* 224:899-904 (1992) and de Vos, et al., *Science* 255:306-312 (1992)).

20 The Ig derived proteins or specified portions or variants of the present invention, or specified variants thereof, can comprise any number of contiguous amino acid residues from an Ig derived protein or specified portion or variant of the present invention, wherein that number is

selected from the group of integers consisting of from 10-100% of the number of contiguous residues in a RELP Ig derived protein or specified portion or variant. Optionally, this subsequence of contiguous amino acids is at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250 or more amino acids in length, or any range or value therein. Further, the number of such subsequences can be any integer selected from the group consisting of from 1 to 20, such as at least 2, 3, 4, or 5.

As those of skill will appreciate, the present invention includes at least one biologically active Ig derived protein or specified portion or variant of the present invention. Biologically active Ig derived proteins or specified portions or variants have a specific activity at least 20%, 30%, or 40%, and preferably at least 50%, 60%, or 70%, and most preferably at least 80%, 90%, or 95%-1000% of that of the native (non-synthetic), endogenous or related and known Ig derived protein or specified portion or variant. Methods of assaying and quantifying measures of enzymatic activity and substrate specificity, are well known to those of skill in the art.

In another aspect, the invention relates to human Ig derived proteins and antigen-binding fragments, as described herein, which are modified by the covalent attachment of an organic moiety.

The Ig dervied proteins of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to Ig dervied proteins and delivered in vivo to sites of antigen include ²¹²Bi, ¹³¹I, ¹⁸⁶Re, and ⁹⁰Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to Ig dervied proteins and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A.G., et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed., Macmillan Publishing Co., 1985.

The Ig dervied proteins of this invention may be advantageously utilized in combination with other monoclonal or murine and chimeric Ig dervied proteins, fragments and regions , or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the Ig dervied proteins.

Such modification can produce an Ig derived protein or antigen-binding fragment

with improved pharmacokinetic properties (e.g., increased *in vivo* serum half-life). The organic moiety can be a linear or branched hydrophilic polymeric group, fatty acid group, or fatty acid ester group. In particular embodiments, the hydrophilic polymeric group can have a molecular weight of about 800 to about 120,000 Daltons and can be a polyalkane glycol (e.g., 5 polyethylene glycol (PEG), polypropylene glycol (PPG)), carbohydrate polymer, amino acid polymer or polyvinyl pyrrolidone, and the fatty acid or fatty acid ester group can comprise from about eight to about forty carbon atoms.

The modified Ig derived proteins and antigen-binding fragments of the invention can comprise one or more organic moieties that are covalently bonded, directly or indirectly, to the 10 Ig derived protein or specified portion or variant. Each organic moiety that is bonded to an Ig derived protein or antigen-binding fragment of the invention can independently be a hydrophilic polymeric group, a fatty acid group or a fatty acid ester group. As used herein, the term “fatty acid” encompasses mono-carboxylic acids and di-carboxylic acids. A “hydrophilic 15 polymeric group,” as the term is used herein, refers to an organic polymer that is more soluble in water than in octane. For example, polylysine is more soluble in water than in octane. Thus, an Ig derived protein modified by the covalent attachment of polylysine is encompassed by the invention. Hydrophilic polymers suitable for modifying Ig derived proteins of the invention can be linear or branched and include, for example, polyalkane glycols (e.g., PEG, monomethoxy-polyethylene glycol (mPEG), PPG and the like), carbohydrates (e.g., dextran, 20 cellulose, oligosaccharides, polysaccharides and the like), polymers of hydrophilic amino acids (e.g., polylysine, polyarginine, polyaspartate and the like), polyalkane oxides (e.g., polyethylene oxide, polypropylene oxide and the like) and polyvinyl pyrrolidone. Preferably, the hydrophilic polymer that modifies the Ig derived protein of the invention has a molecular weight of about 800 to about 150,000 Daltons as a separate molecular entity. For example 25 PEG₅₀₀₀ and PEG_{20,000}, wherein the subscript is the average molecular weight of the polymer in Daltons, can be used.

The hydrophilic polymeric group can be substituted with one to about six alkyl, fatty acid or fatty acid ester groups. Hydrophilic polymers that are substituted with a fatty acid or fatty acid ester group can be prepared by employing suitable methods. For example, a polymer comprising an amine group can be coupled to a carboxylate of the fatty acid or fatty acid ester, and an activated carboxylate (e.g., activated with N, N-carbonyl diimidazole) on a fatty acid or fatty acid ester can be coupled to a hydroxyl group on a polymer. 30

Fatty acids and fatty acid esters suitable for modifying Ig derived proteins of the invention can be saturated or can contain one or more units of unsaturation. Fatty acids that

are suitable for modifying Ig derived proteins of the invention include, for example, n-dodecanoate (C₁₂, laurate), n-tetradecanoate (C₁₄, myristate), n-octadecanoate (C₁₈, stearate), n-eicosanoate (C₂₀, arachidate), n-docosanoate (C₂₂, behenate), n-triacontanoate (C₃₀), n-tetracontanoate (C₄₀), *cis*- 9-octadecanoate (C₁₈, oleate), all

5 *cis*- 5,8,11,14-eicosatetraenoate (C₂₀, arachidonate), octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like. Suitable fatty acid esters include mono-esters of dicarboxylic acids that comprise a linear or branched lower alkyl group. The lower alkyl group can comprise from one to about twelve, preferably one to about six, carbon atoms.

10 The modified human Ig derived proteins and antigen-binding fragments can be prepared using suitable methods, such as by reaction with one or more modifying agents. A "modifying agent" as the term is used herein, refers to a suitable organic group (e.g., hydrophilic polymer, a fatty acid, a fatty acid ester) that comprises an activating group. An "activating group" is a chemical moiety or functional group that can, under appropriate conditions, react with a second chemical group thereby forming a covalent bond between the modifying agent and the second chemical group. For example, amine-reactive activating groups include electrophilic groups such as tosylate, mesylate, halo (chloro, bromo, fluoro, iodo), N-hydroxysuccinimidyl esters (NHS), and the like. Activating groups that can react with thiols include, for example, maleimide, iodoacetyl, acryloyl, pyridyl disulfides, 5-thiol-2-nitrobenzoic acid thiol (TNB-thiol), and the like. An aldehyde functional group can be coupled to amine- or hydrazide-containing molecules, and an azide group can react with a trivalent phosphorous group to form phosphoramidate or phosphorimide linkages. Suitable methods to introduce activating groups into molecules are known in the art (see for example, Hermanson, G. T., *Bioconjugate Techniques*, Academic Press: San Diego, CA (1996)). An activating group can be bonded directly to the organic group (e.g., hydrophilic polymer, fatty acid, fatty acid ester), or through a linker moiety, for example a divalent C₁-C₁₂ group wherein one or more carbon atoms can be replaced by a heteroatom such as oxygen, nitrogen or sulfur. Suitable linker moieties include, for example, tetraethylene glycol, -(CH₂)₃-, -NH-(CH₂)₆-NH-, -(CH₂)₂-NH- and -CH₂-O-CH₂-CH₂-O-CH₂-CH₂-O-CH-NH-. Modifying agents that comprise a linker moiety can be produced, for example, by reacting a mono-Boc-alkyldiamine (e.g., mono-Boc-ethylenediamine, mono-Boc-diaminohexane) with a fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to form an amide bond between the free amine and the fatty acid carboxylate. The Boc protecting group can be removed from the product by treatment with trifluoroacetic acid (TFA) to expose a primary amine that can be

RELP Ig derived protein or specified portion or variant compositions

coupled to another carboxylate as described, or can be reacted with maleic anhydride and the resulting product cyclized to produce an activated maleimido derivative of the fatty acid. (See, for example, Thompson, *et al.*, WO 92/16221 the entire teachings of which are incorporated herein by reference.)

5 The modified Ig derived proteins of the invention can be produced by reacting a human Ig derived protein or antigen-binding fragment with a modifying agent. For example, the organic moieties can be bonded to the Ig derived protein in a non-site specific manner by employing an amine-reactive modifying agent, for example, an NHS ester of PEG. Modified human Ig derived proteins or antigen-binding fragments can also be prepared by reducing
10 disulfide bonds (e.g., intra-chain disulfide bonds) of an Ig derived protein or antigen-binding fragment. The reduced Ig derived protein or antigen-binding fragment can then be reacted with a thiol-reactive modifying agent to produce the modified Ig derived protein of the invention. Modified human Ig derived proteins and antigen-binding fragments comprising an organic moiety that is bonded to specific sites of an Ig derived protein or specified portion or
15 variant of the present invention can be prepared using suitable methods, such as reverse proteolysis (Fisch *et al.*, *Bioconjugate Chem.*, 3:147-153 (1992); Werlen *et al.*, *Bioconjugate Chem.*, 5:411-417 (1994); Kumaran *et al.*, *Protein Sci.* 6(10):2233-2241 (1997); Itoh *et al.*, *Bioorg. Chem.*, 24(1): 59-68 (1996); Capellas *et al.*, *Biotechnol. Bioeng.*, 56(4):456-463 (1997)), and the methods described in Hermanson, G. T., *Bioconjugate Techniques*, Academic Press: San Diego, CA (1996).

RELP Ig derived protein OR SPECIFIED PORTION OR VARIANT COMPOSITIONS

The present invention also provides at least one RELP Ig derived protein or specified portion or variant composition comprising at least one, at least two, at least three, at least four, at least five, at least six or more RELP Ig derived proteins or specified portions or variants thereof, as described herein and/or as known in the art that are provided in a non-naturally occurring composition, mixture or form. Such compositions comprise non-naturally occurring compositions comprising at least one or two full length, C- and/or N-terminally deleted variants, domains, fragments, or specified variants, of the RELP Ig derived protein amino acid sequence. Such composition percentages are by weight, volume, concentration, molarity, or molality as liquid or dry solutions, mixtures, suspension, emulsions or colloids, as known in the art or as described herein.

RELP Ig derived protein or specified portion or variant compositions of the present invention can further comprise at least one of any suitable auxiliary, such as, but not limited to, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like.

Pharmaceutically acceptable auxiliaries are preferred. Non-limiting examples of, and methods of preparing such sterile solutions are well known in the art, such as, but limited to, Gennaro, Ed., *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co. (Easton, PA) 1990. Pharmaceutically acceptable carriers can be routinely selected that are suitable for the mode of administration, solubility and/or stability of the RELP protein composition as well known in the art or as described herein.

Pharmaceutical excipients and additives useful in the present composition include but are not limited to proteins, peptides, amino acids, lipids, and carbohydrates (e.g., sugars, including monosaccharides, di-, tri-, tetra-, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid/Ig derived protein or specified portion or variant components, which can also function in a buffering capacity, include alanine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. One preferred amino acid is glycine.

Carbohydrate excipients suitable for use in the invention include, for example, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol), myoinositol and the like. Preferred carbohydrate excipients for use in the present invention are mannitol, trehalose, and raffinose.

RELP protein compositions can also include a buffer or a pH adjusting agent; typically, the buffer is a salt prepared from an organic acid or base. Representative buffers include organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers. Preferred buffers for use in the present compositions are organic acid salts such as citrate.

Additionally, the RELP Ig derived protein or specified portion or variant compositions of the invention can include polymeric excipients/additives such as polyvinylpyrrolidones, ficolls (a polymeric sugar), dextrans (e.g., cyclodextrins, such as 2-hydroxypropyl-β-

cyclodextrin), polyethylene glycols, flavoring agents, antimicrobial agents, sweeteners, antioxidants, antistatic agents, surfactants (e.g., polysorbates such as "TWEEN 20" and "TWEEN 80"), lipids (e.g., phospholipids, fatty acids), steroids (e.g., cholesterol), and chelating agents (e.g., EDTA).

5 These and additional known pharmaceutical excipients and/or additives suitable for use in the RELP protein compositions according to the invention are known in the art, e.g., as listed in "Remington: The Science & Practice of Pharmacy", 19th ed., Williams & Williams, (1995), and in the "Physician's Desk Reference", 52nd ed., Medical Economics, Montvale, NJ (1998), the disclosures of which are entirely incorporated herein by reference. Preferred
10 carrier or excipient materials are carbohydrates (e.g., saccharides and alditols) and buffers (e.g., citrate) or polymeric agents.

Formulations

As noted above, the invention provides for stable formulations, which is preferably a phosphate buffer with saline or a chosen salt, as well as preserved solutions and
15 formulations containing a preservative as well as multi-use preserved formulations suitable for pharmaceutical or veterinary use, comprising at least one RELP Ig derived protein or specified portion or variant in a pharmaceutically acceptable formulation. Preserved formulations contain at least one known preservative or optionally selected from the group consisting of at least one phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, phenylmercuric
20 nitrite, phenoxyethanol, formaldehyde, chlorobutanol, magnesium chloride (e.g., hexahydrate), alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in an aqueous diluent. Any suitable concentration or mixture can be used as known in the art, such as 0.001-5%, or any range or value therein, such as, but not limited to 0.001, 0.003, 0.005, 0.009, 0.01, 0.02,
25 0.03, 0.05, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0,
4.3, 4.5, 4.6, 4.7, 4.8, 4.9, or any range or value therein. Non-limiting examples include, no
preservative, 0.1-2% m-cresol (e.g., 0.2, 0.3, 0.4, 0.5, 0.9, 1.0%), 0.1-3% benzyl alcohol (e.g.,
0.5, 0.9, 1.1, 1.5, 1.9, 2.0, 2.5%), 0.001-0.5% thimerosal (e.g., 0.005, 0.01), 0.001-2.0%
30 phenol (e.g., 0.05, 0.25, 0.28, 0.5, 0.9, 1.0%), 0.0005-1.0% alkylparaben(s) (e.g., 0.00075, 0.0009, 0.001, 0.002, 0.005, 0.0075, 0.009, 0.01, 0.02, 0.05, 0.075, 0.09, 0.1, 0.2, 0.3, 0.5, 0.75, 0.9, 1.0%), and the like.

As noted above, the invention provides an article of manufacture, comprising packaging material and at least one vial comprising a solution of at least one RELP Ig derived

protein or specified portion or variant with the prescribed buffers and/or preservatives, optionally in an aqueous diluent, wherein said packaging material comprises a label that indicates that such solution can be held over a period of 1, 2, 3, 4, 5, 6, 9, 12, 18, 20, 24, 30, 36, 40, 48, 54, 60, 66, 72 hours or greater. The invention further comprises an article of manufacture, comprising packaging material, a first vial comprising lyophilized at least one RELP Ig derived protein or specified portion or variant, and a second vial comprising an aqueous diluent of prescribed buffer or preservative, wherein said packaging material comprises a label that instructs a patient to reconstitute the at least one RELP Ig derived protein or specified portion or variant in the aqueous diluent to form a solution that can be held over a period of twenty-four hours or greater.

The at least one RELP proteinIg derived protein or specified portion or variant used in accordance with the present invention can be produced by recombinant means, including from mammalian cell or transgenic preparations, or can be purified from other biological sources, as described herein or as known in the art.

The range of at least one RELP Ig derived protein or specified portion or variant in the product of the present invention includes amounts yielding upon reconstitution, if in a wet/dry system, concentrations from about 1.0 µg/ml to about 1000 mg/ml, although lower and higher concentrations are operable and are dependent on the intended delivery vehicle, e.g., solution formulations will differ from transdermal patch, pulmonary, transmucosal, or osmotic or micro pump methods.

Preferably, the aqueous diluent optionally further comprises a pharmaceutically acceptable preservative. Preferred preservatives include those selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof. The concentration of preservative used in the formulation is a concentration sufficient to yield an anti-microbial effect. Such concentrations are dependent on the preservative selected and are readily determined by the skilled artisan.

Other excipients, e.g. isotonicity agents, buffers, antioxidants, preservative enhancers, can be optionally and preferably added to the diluent. An isotonicity agent, such as glycerin, is commonly used at known concentrations. A physiologically tolerated buffer is preferably added to provide improved pH control. The formulations can cover a wide range of pHs, such as from about pH 4 to about pH 10, and preferred ranges from about pH 5 to about pH 9, and a most preferred range of about 6.0 to about 8.0. Preferably the formulations of the present

invention have pH between about 6.8 and about 7.8. Preferred buffers include phosphate buffers, most preferably sodium phosphate, particularly phosphate buffered saline (PBS).

Other additives, such as a pharmaceutically acceptable solubilizers like Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 80 (polyoxyethylene (20) sorbitan monooleate), Pluronic F68 (polyoxyethylene polyoxypropylene block copolymers), and PEG (polyethylene glycol) or non-ionic surfactants such as polysorbate 20 or 80 or poloxamer 184 or 188, Pluronic® polyols, other block co-polymers, and chelators such as EDTA and EGTA can optionally be added to the formulations or compositions to reduce aggregation. These additives are particularly useful if a pump or plastic container is used to administer the formulation. The presence of pharmaceutically acceptable surfactant mitigates the propensity for the protein to aggregate.

The formulations of the present invention can be prepared by a process which comprises mixing at least one RELP Ig derived protein or specified portion or variant and a preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben, (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal or mixtures thereof in an aqueous diluent. Mixing the at least one RELP Ig derived protein or specified portion or variant and preservative in an aqueous diluent is carried out using conventional dissolution and mixing procedures. To prepare a suitable formulation, for example, a measured amount of at least one RELP Ig derived protein or specified portion or variant in buffered solution is combined with the desired preservative in a buffered solution in quantities sufficient to provide the protein and preservative at the desired concentrations. Variations of this process would be recognized by one of ordinary skill in the art. For example, the order the components are added, whether additional additives are used, the temperature and pH at which the formulation is prepared, are all factors that may be optimized for the concentration and means of administration used.

The claimed formulations can be provided to patients as clear solutions or as dual vials comprising a vial of lyophilized at least one RELP Ig derived protein or specified portion or variant that is reconstituted with a second vial containing water, a preservative and/or excipients, preferably a phosphate buffer and/or saline and a chosen salt, in an aqueous diluent. Either a single solution vial or dual vial requiring reconstitution can be reused multiple times and can suffice for a single or multiple cycles of patient treatment and thus can provide a more convenient treatment regimen than currently available.

The present claimed articles of manufacture are useful for administration over

a period of immediately to twenty-four hours or greater. Accordingly, the presently claimed articles of manufacture offer significant advantages to the patient. Formulations of the invention can optionally be safely stored at temperatures of from about 2 to about 40°C and retain the biologically activity of the protein for extended periods of time, thus, allowing a
5 package label indicating that the solution can be held and/or used over a period of 6, 12, 18, 24, 36, 48, 72, or 96 hours or greater. If preserved diluent is used, such label can include use up to 1-12 months, one-half, one and a half, and/or two years.

The solutions of at least one RELP Ig derived protein or specified portion or variant in the invention can be prepared by a process that comprises mixing at least one Ig
10 derived protein or specified portion or variant in an aqueous diluent. Mixing is carried out using conventional dissolution and mixing procedures. To prepare a suitable diluent, for example, a measured amount of at least one Ig derived protein or specified portion or variant in water or buffer is combined in quantities sufficient to provide the protein and optionally a preservative or buffer at the desired concentrations. Variations of this process would be
15 recognized by one of ordinary skill in the art. For example, the order the components are added, whether additional additives are used, the temperature and pH at which the formulation is prepared, are all factors that may be optimized for the concentration and means of administration used.

The claimed products can be provided to patients as clear solutions or as dual
20 vials comprising a vial of lyophilized at least one RELP Ig derived protein or specified portion or variant that is reconstituted with a second vial containing the aqueous diluent. Either a single solution vial or dual vial requiring reconstitution can be reused multiple times and can suffice for a single or multiple cycles of patient treatment and thus provides a more convenient treatment regimen than currently available.

The claimed products can be provided indirectly to patients by providing to pharmacies, clinics, or other such institutions and facilities, clear solutions or dual vials
25 comprising a vial of lyophilized at least one RELP Ig derived protein or specified portion or variant that is reconstituted with a second vial containing the aqueous diluent. The clear solution in this case can be up to one liter or even larger in size, providing a large reservoir from which smaller portions of the at least one Ig derived protein or specified portion or
30 variant solution can be retrieved one or multiple times for transfer into smaller vials and provided by the pharmacy or clinic to their customers and/or patients.

Recognized devices comprising these single vial systems include those pen-injector devices for delivery of a solution such as BD Pens, BD Autojector®, Humaject®,

NovoPen®, B-D®Pen, AutoPen®, and OptiPen®, GenotropinPen®, Genotronorm Pen®, Humatro Pen®, Reco-Pen®, Roferon Pen®, Biojector®, iject®, J-tip Needle-Free Injector®, Inraject®, Medi-Ject®, e.g., as made or developed by Becton Dickensen (Franklin Lakes, NJ, www.bectondickenson.com), Disetronic (Burgdorf, Switzerland, www.disetronic.com; Bioject, 5 Portland, Oregon (www.bioject.com); National Medical Products , Weston Medical (Peterborough, UK, www.weston-medical.com), Medi-Ject Corp (Minneapolis, MN, www.mediject.com). Recognized devices comprising a dual vial system include those pen-injector systems for reconstituting a lyophilized drug in a cartridge for delivery of the reconstituted solution such as the HumatroPen®.

10 The products presently claimed include packaging material. The packaging material provides, in addition to the information required by the regulatory agencies, the conditions under which the product can be used. The packaging material of the present invention provides instructions to the patient to reconstitute the at least one RELP Ig derived protein or specified portion or variant in the aqueous diluent to form a solution and to use the 15 solution over a period of 2-24 hours or greater for the two vial, wet/dry, product. For the single vial, solution product, the label indicates that such solution can be used over a period of 2-24 hours or greater. The presently claimed products are useful for human pharmaceutical product use.

20 The formulations of the present invention can be prepared by a process that comprises mixing at least one RELP Ig derived protein or specified portion or variant and a selected buffer, preferably a phosphate buffer containing saline or a chosen salt. Mixing the at least one Ig derived protein or specified portion or variant and buffer in an aqueous diluent is carried out using conventional dissolution and mixing procedures. To prepare a suitable 25 formulation, for example, a measured amount of at least one Ig derived protein or specified portion or variant in water or buffer is combined with the desired buffering agent in water in quantities sufficient to provide the protein and buffer at the desired concentrations. Variations of this process would be recognized by one of ordinary skill in the art. For example, the order the components are added, whether additional additives are used, the temperature and pH at which the formulation is prepared, are all factors that can be optimized for the concentration 30 and means of administration used.

The claimed stable or preserved formulations can be provided to patients as clear solutions or as dual vials comprising a vial of lyophilized at least one RELP Ig derived protein or specified portion or variant that is reconstituted with a second vial containing a preservative or buffer and excipients in an aqueous diluent. Either a single solution vial or

dual vial requiring reconstitution can be reused multiple times and can suffice for a single or multiple cycles of patient treatment and thus provides a more convenient treatment regimen than currently available.

At least one RELP Ig derived protein or specified portion or variant in either the stable
5 or preserved formulations or solutions described herein, can be administered to a patient in accordance with the present invention via a variety of delivery methods including SC or IM injection; transdermal, pulmonary, transmucosal, implant, osmotic pump, cartridge, micro pump, or other means appreciated by the skilled artisan, as well-known in the art.

Therapeutic Applications

The present invention also provides a method for modulating or treating at least one malignant disease in a cell, tissue, organ, animal or patient, including, but not limited to, at least one of: leukemia, acute leukemia, acute lymphoblastic leukemia (ALL), B-cell, T-cell or FAB ALL, acute myeloid leukemia (AML), chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), hairy cell leukemia, myelodysplastic syndrome (MDS), a lymphoma, Hodgkin's disease, a malignant lymphoma, non-hodgkin's lymphoma, Burkitt's lymphoma, multiple myeloma, Kaposi's sarcoma, colorectal carcinoma, pancreatic carcinoma, nasopharyngeal carcinoma, malignant histiocytosis, paraneoplastic syndrome/hypercalcemia of malignancy, solid tumors, adenocarcinomas, sarcomas, malignant melanoma, and the like.

Any method of the present invention can comprise administering an effective amount
20 of a composition or pharmaceutical composition comprising at least one RELP Ig derived protein or specified portion or variant to a cell, tissue, organ, animal or patient in need of such modulation, treatment or therapy. Such a method can optionally further comprise co-administration or combination therapy for treating such immune diseases, wherein the administering of said at least one RELP Ig derived protein, specified portion or variant thereof,
25 further comprises administering, before concurrently, and/or after, at least one selected from at least one TNF antagonist (e.g., but not limited to a TNF Ig derived protein or fragment, a soluble TNF receptor or fragment, fusion proteins thereof, or a small molecule TNF antagonist), an antirheumatic, a muscle relaxant, a narcotic, a non-steroid anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial (e.g., aminoglycoside, an antifungal, an antiparasitic, an antiviral, a carbapenem, cephalosporin, a fluroquinolone, a macrolide, a penicillin, a sulfonamide, a tetracycline, another antimicrobial), an antipsoriatic, a corticosteroid, an anabolic steroid, a diabetes related agent, a mineral, a nutritional, a thyroid agent, a vitamin, a calcium related hormone, an antidiarrheal, an antitussive, an antiemetic, an antiulcer, a laxative, an

anticoagulant, an erythropoietin (e.g., epoetin alpha), a filgrastim (e.g., G-CSF, Neupogen), a sargramostim (GM-CSF, Leukine), an immunization, an immunoglobulin, an immunosuppressive (e.g., basiliximab, cyclosporine, daclizumab), a growth hormone, a hormone replacement drug, an estrogen receptor modulator, a mydriatic, a cycloplegic, an
5 alkylating agent, an antimetabolite, a mitotic inhibitor, a radiopharmaceutical, an antidepressant, antimanic agent, an antipsychotic, an anxiolytic, a hypnotic, a sympathomimetic, a stimulant, donepezil, tacrine, an asthma medication, a beta agonist, an inhaled steroid, a leukotriene inhibitor, a methylxanthine, a cromolyn, an epinephrine or analog, dornase alpha (Pulmozyme), a cytokine or a cytokine antagonist. Suitable dosages
10 are well known in the art. See, e.g., Wells et al., eds., *Pharmacotherapy Handbook*, 2nd Edition, Appleton and Lange, Stamford, CT (2000); *PDR Pharmacopoeia*, Tarascon Pocket *Pharmacopoeia* 2000, Deluxe Edition, Tarascon Publishing, Loma Linda, CA (2000), each of which references are entirely incorporated herein by reference.

TNF antagonists suitable for compositions, combination therapy, co-administration,
15 devices and/or methods of the present invention (further comprising at least one anti body, specified portion and variant thereof, of the present invention), include, but are not limited to, anti-TNF Ig derived proteins, antigen-binding fragments thereof, and receptor molecules which bind specifically to TNF; compounds which prevent and/or inhibit TNF synthesis, TNF release or its action on target cells, such as thalidomide, tenidap, phosphodiesterase inhibitors
20 (e.g, pentoxifylline and rolipram), A2b adenosine receptor agonists and A2b adenosine receptor enhancers; compounds which prevent and/or inhibit TNF receptor signalling, such as mitogen activated protein (MAP) kinase inhibitors; compounds which block and/or inhibit membrane TNF cleavage, such as metalloproteinase inhibitors; compounds which block and/or inhibit TNF activity, such as angiotensin converting enzyme (ACE) inhibitors (e.g., captopril);
25 and compounds which block and/or inhibit TNF production and/or synthesis, such as MAP kinase inhibitors.

As used herein, a "tumor necrosis factor Ig derived protein," "TNF Ig derived protein," "TNF Ig derived protein," or fragment and the like decreases, blocks, inhibits, abrogates or interferes with TNF activity *in vitro*, *in situ* and/or preferably *in vivo*. For example, a
30 suitable TNF human Ig derived protein of the present invention can bind TNF and includes anti-TNF Ig derived proteins, antigen-binding fragments thereof, and specified mutants or domains thereof that bind specifically to TNF. A suitable TNF antibody or fragment can also decrease block, abrogate, interfere, prevent and/or inhibit TNF RNA, DNA or protein synthesis, TNF release, TNF receptor signaling, membrane TNF cleavage, TNF activity, TNF

production and/or synthesis.

Chimeric Ig derived protein cA2 consists of the antigen binding variable region of the high-affinity neutralizing mouse anti-human TNF IgG1 Ig derived protein, designated A2, and the constant regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fc region improves allogeneic Ig derived protein effector function, increases the circulating serum half-life and decreases the immunogenicity of the Ig derived protein. The avidity and epitope specificity of the chimeric Ig derived protein cA2 is derived from the variable region of the murine Ig derived protein A2. In a particular embodiment, a preferred source for nucleic acids encoding the variable region of the murine Ig derived protein A2 is the A2 hybridoma cell line.

Chimeric A2 (cA2) neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner. From binding assays of chimeric Ig derived protein cA2 and recombinant human TNF, the affinity constant of chimeric Ig derived protein cA2 was calculated to be $1.04 \times 10^{10} M^{-1}$. Preferred methods for determining monoclonal Ig derived protein specificity and affinity by competitive inhibition can be found in Harlow, *et al.*, *Ig derived proteins: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988; Colligan *et al.*, eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, New York, (1992-2000); Kozbor *et al.*, *Immunol. Today*, 4:72-79 (1983); Ausubel *et al.*, eds. *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1987-2000); and Muller, *Meth. Enzymol.*, 92:589-601 (1983), which references are entirely incorporated herein by reference.

In a particular embodiment, murine monoclonal Ig derived protein A2 is produced by a cell line designated c134A. Chimeric Ig derived protein cA2 is produced by a cell line designated c168A.

Additional examples of monoclonal anti-TNF Ig derived proteins that can be used in the present invention are described in the art (see, e.g., U.S. Patent No. 5,231,024; Möller, A. *et al.*, *Cytokine* 2(3):162-169 (1990); U.S. Application No. 07/943,852 (filed September 11, 1992); Rathjen *et al.*, International Publication No. WO 91/02078 (published February 21, 1991); Rubin *et al.*, EPO Patent Publication No. 0 218 868 (published April 22, 1987); Yone *et al.*, EPO Patent Publication No. 0 288 088 (October 26, 1988); Liang, *et al.*, *Biochem. Biophys. Res. Comm.* 137:847-854 (1986); Meager, *et al.*, *Hybridoma* 6:305-311 (1987); Fendly *et al.*, *Hybridoma* 6:359-369 (1987); Bringman, *et al.*, *Hybridoma* 6:489-507 (1987); and Hirai, *et al.*, *J. Immunol. Meth.* 96:57-62 (1987), which references are entirely incorporated herein by reference).

TNF Receptor Molecules

Preferred TNF receptor molecules useful in the present invention are those that bind TNF with high affinity (see, e.g., Feldmann *et al.*, International Publication No. WO 92/07076 (published April 30, 1992); Schall *et al.*, *Cell* 61:361-370 (1990); and Loetscher *et al.*, *Cell* 61:351-359 (1990), which references are entirely incorporated herein by reference) and optionally possess low immunogenicity. In particular, the 55 kDa (p55 TNF-R) and the 75 kDa (p75 TNF-R) TNF cell surface receptors are useful in the present invention. Truncated forms of these receptors, comprising the extracellular domains (ECD) of the receptors or functional portions thereof (see, e.g., Corcoran *et al.*, *Eur. J. Biochem.* 223:831-840 (1994)), are also useful in the present invention. Truncated forms of the TNF receptors, comprising the ECD, have been detected in urine and serum as 30 kDa and 40 kDa TNF inhibitory binding proteins (Engelmann, H. *et al.*, *J. Biol. Chem.* 265:1531-1536 (1990)). TNF receptor multimeric molecules and TNF immunoreceptor fusion molecules, and derivatives and fragments or portions thereof, are additional examples of TNF receptor molecules which are useful in the methods and compositions of the present invention. The TNF receptor molecules which can be used in the invention are characterized by their ability to treat patients for extended periods with good to excellent alleviation of symptoms and low toxicity. Low immunogenicity and/or high affinity, as well as other undefined properties, may contribute to the therapeutic results achieved.

TNF receptor multimeric molecules useful in the present invention comprise all or a functional portion of the ECD of two or more TNF receptors linked via one or more polypeptide linkers or other nonpeptide linkers, such as polyethylene glycol (PEG). The multimeric molecules can further comprise a signal peptide of a secreted protein to direct expression of the multimeric molecule. These multimeric molecules and methods for their production have been described in U.S. Application No. 08/437,533 (filed May 9, 1995), the content of which is entirely incorporated herein by reference.

TNF immunoreceptor fusion molecules useful in the methods and compositions of the present invention comprise at least one portion of one or more immunoglobulin molecules and all or a functional portion of one or more TNF receptors. These immunoreceptor fusion molecules can be assembled as monomers, or hetero- or homo-multimers. The immunoreceptor fusion molecules can also be monovalent or multivalent. An example of such a TNF immunoreceptor fusion molecule is TNF receptor/IgG fusion protein. TNF immunoreceptor fusion molecules and methods for their production have been described in the art (Lesslauer *et al.*, *Eur. J. Immunol.* 21:2883-2886 (1991); Ashkenazi *et al.*, *Proc. Natl.*

Acad. Sci. USA 88:10535-10539 (1991); Peppel *et al.*, *J. Exp. Med.* 174:1483-1489 (1991); Kolls *et al.*, *Proc. Natl. Acad. Sci. USA* 91:215-219 (1994); Butler *et al.*, *Cytokine* 6(6):616-623 (1994); Baker *et al.*, *Eur. J. Immunol.* 24:2040-2048 (1994); Beutler *et al.*, U.S. Patent No. 5,447,851; and U.S. Application No. 08/442,133 (filed May 16, 1995), each of which references are entirely incorporated herein by reference). Methods for producing immunoreceptor fusion molecules can also be found in Capon *et al.*, U.S. Patent No. 5,116,964; Capon *et al.*, U.S. Patent No. 5,225,538; and Capon *et al.*, *Nature* 337:525-531 (1989), which references are entirely incorporated herein by reference.

A functional equivalent, derivative, fragment or region of TNF receptor molecule refers to the portion of the TNF receptor molecule, or the portion of the TNF receptor molecule sequence which encodes TNF receptor molecule, that is of sufficient size and sequences to functionally resemble TNF receptor molecules that can be used in the present invention (e.g., bind TNF with high affinity and possess low immunogenicity). A functional equivalent of TNF receptor molecule also includes modified TNF receptor molecules that functionally resemble TNF receptor molecules that can be used in the present invention (e.g., bind TNF with high affinity and possess low immunogenicity). For example, a functional equivalent of TNF receptor molecule can contain a "SILENT" codon or one or more amino acid substitutions, deletions or additions (e.g., substitution of one acidic amino acid for another acidic amino acid; or substitution of one codon encoding the same or different hydrophobic amino acid for another codon encoding a hydrophobic amino acid). See Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience, New York (1987-2000).

Cytokines include any known cytokine. See, e.g., CopewithCytokines.com. Cytokine antagonists include, but are not limited to, any Ig derived protein, fragment or mimetic, any soluble receptor, fragment or mimetic, any small molecule antagonist, or any combination thereof.

Therapeutic Treatments. Any method of the present invention can comprise a method for treating a RELP protein mediated disorder, comprising administering an effective amount of a composition or pharmaceutical composition comprising at least one RELP Ig derived protein or specified portion or variant to a cell, tissue, organ, animal or patient in need of such modulation, treatment or therapy. Such a method can optionally further comprise co-administration or combination therapy for treating such immune diseases, wherein the administering of said at least one RELP Ig derived protein, specified portion or variant thereof, further comprises administering, before concurrently, and/or after, at least one selected from at

least one TNF antagonist (e.g., but not limited to a TNF Ig derived protein or fragment, a soluble TNF receptor or fragment, fusion proteins thereof, or a small molecule TNF antagonist), an antirheumatic, a muscle relaxant, a narcotic, a non-steroid anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial (e.g., aminoglycoside, an antifungal, an antiparasitic, an antiviral, a carbapenem, cephalosporin, a flurorquinolone, a macrolide, a penicillin, a sulfonamide, a tetracycline, another antimicrobial), an antipsoriatic, a corticosteroid, an anabolic steroid, a diabetes related agent, a mineral, a nutritional, a thyroid agent, a vitamin, a calcium related hormone, an antidiarrheal, an antitussive, an antiemetic, an antiulcer, a laxative, an anticoagulant, an erythropoietin (e.g., epoetin alpha), a filgrastim (e.g., G-CSF, Neupogen), a sargramostim (GM-CSF, Leukine), an immunization, an immunoglobulin, an immunosuppressive (e.g., basiliximab, cyclosporine, daclizumab), a growth hormone, a hormone replacement drug, an estrogen receptor modulator, a mydriatic, a cycloplegic, an alkylating agent, an antimetabolite, a mitotic inhibitor, a radiopharmaceutical, an antidepressant, antimanic agent, an antipsychotic, an anxiolytic, a hypnotic, a sympathomimetic, a stimulant, donepezil, tacrine, an asthma medication, a beta agonist, an inhaled steroid, a leukotriene inhibitor, a methylxanthine, a cromolyn, an epinephrine or analog, dornase alpha (Pulmozyme), a cytokine or a cytokine antagonist. Suitable dosages are well known in the art. See, e.g., Wells et al., eds., *Pharmacotherapy Handbook*, 2nd Edition, Appleton and Lange, Stamford, CT (2000); *PDR Pharmacopoeia*, Tarascon Pocket Pharmacopoeia 2000, Deluxe Edition, Tarascon Publishing, Loma Linda, CA (2000), each of which references are entirely incorporated herein by reference.

Typically, treatment of pathologic conditions is effected by administering an effective amount or dosage of at least one RELP protein composition that total, on average, a range from at least about 0.01 to 500 milligrams of at least one RELP protein Ig derived protein or specified portion or variant /kilogram of patient per dose, and preferably from at least about 0.1 to 100 milligrams Ig derived protein or specified portion or variant /kilogram of patient per single or multiple administration, depending upon the specific activity of contained in the composition. Alternatively, the effective serum concentration can comprise 0.1-5000 µg/ml serum concentration per single or multiple administration. Suitable dosages are known to medical practitioners and will, of course, depend upon the particular disease state, specific activity of the composition being administered, and the particular patient undergoing treatment. In some instances, to achieve the desired therapeutic amount, it can be necessary to provide for repeated administration, *i.e.*, repeated individual administrations of a particular monitored or metered

dose, where the individual administrations are repeated until the desired daily dose or effect is achieved.

Preferred doses can optionally include 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 and/or 100 mg/kg/administration, or any range, value or fraction thereof, or to achieve a serum concentration of 0.1, 0.5, 0.9, 1.0, 1.1, 1.2, 1.5, 1.9, 2.0, 2.5, 2.9, 3.0, 3.5, 3.9, 4.0, 4.5, 4.9, 5.0, 5.5, 5.9, 6.0, 6.5, 6.9, 7.0, 7.5, 7.9, 8.0, 8.5, 8.9, 9.0, 9.5, 9.9, 10, 10.5, 10.9, 11, 11.5, 11.9, 20, 12.5, 12.9, 13.0, 13.5, 13.9, 14.0, 14.5, 4.9, 5.0, 5.5., 5.9, 6.0, 6.5, 6.9, 7.0, 7.5, 7.9, 8.0, 8.5, 8.9, 9.0, 9.5, 9.9, 10, 10.5, 10.9, 11, 11.5, 11.9, 12, 12.5, 12.9, 13.0, 13.5, 13.9, 14, 14.5, 15, 15.5, 15.9, 16, 16.5, 16.9, 17, 17.5, 17.9, 18, 18.5, 18.9, 19, 19.5, 19.9, 20, 20.5, 20.9, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 96, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and/or 5000 µg/ml serum concentration per single or multiple administration, or any range, value or fraction thereof.

Alternatively, the dosage administered can vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a dosage of active ingredient can be about 0.1 to 100 milligrams per kilogram of body weight. Ordinarily 0.1 to 50, and preferably 0.1 to 10 milligrams per kilogram per administration or in sustained release form is effective to obtain desired results.

As a non-limiting example, treatment of humans or animals can be provided as a one-time or periodic dosage of at least one Ig derived protein or specified portion or variant of the present invention 0.1 to 100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or alternatively or additionally, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, or 52, or alternatively or additionally, at least one of 1, 2, 3, 4, 5, 6,, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 years, or any combination thereof, using single, infusion or repeated doses.

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit or container. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-99.999% by weight based on the total weight of the composition.

5 For parenteral administration, the Ig derived protein or specified portion or variant can be formulated as a solution, suspension, emulsion or lyophilized powder in association, or separately provided, with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 1-10% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The
10 vehicle or lyophilized powder may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by known or suitable techniques.

Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field.

15 **Alternative Administration**

Many known and developed modes of can be used according to the present invention for administering pharmaceutically effective amounts of at least one RELP Ig derived protein or specified portion or variant according to the present invention. While pulmonary administration is used in the following description, other modes of administration can be used
20 according to the present invention with suitable results.

RELP Ig derived proteins of the present invention can be delivered in a carrier, as a solution, emulsion, colloid, or suspension, or as a dry powder, using any of a variety of devices and methods suitable for administration by inhalation or other modes described here within or known in the art.

25 **PARENTERAL FORMULATIONS AND ADMINISTRATION**

Formulations for parenteral administration can contain as common excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. Aqueous or oily suspensions for injection can be prepared by using an appropriate emulsifier or humidifier and a suspending agent, according to known methods. Agents for injection can be a non-toxic, non-orally administrable diluting agent such as aqueous solution or a sterile injectable solution or suspension in a solvent. As the
30 usable vehicle or solvent, water, Ringer's solution, isotonic saline, etc. are allowed; as an ordinary solvent, or suspending solvent, sterile involatile oil can be used. For these purposes, any kind of involatile oil and fatty acid can be used, including natural or synthetic or

semisynthetic fatty oils or fatty acids; natural or synthetic or semisynthetic mono- or di- or triglycerides. Parental administration is known in the art and includes, but is not limited to, conventional means of injections, a gas pressured needle-less injection device as described in U.S. Pat. No. 5,851,198, and a laser perforator device as described in U.S. Pat. No. 5,839,446

5 entirely incorporated herein by reference.

Alternative Delivery

The invention further relates to the administration of at least one RELP Ig derived protein or specified portion or variant by parenteral, subcutaneous, intramuscular, intravenous, bolus, vaginal, rectal, buccal, sublingual, intranasal, or transdermal means. Protein, Ig derived protein or specified portion or variant compositions can be prepared for use for parenteral (subcutaneous, intramuscular or intravenous) administration particularly in the form of liquid solutions or suspensions; for use in vaginal or rectal administration particularly in semisolid forms such as creams and suppositories; for buccal, or sublingual administration particularly in the form of tablets or capsules; or intranasally particularly in the form of powders, nasal drops or aerosols or certain agents; or transdermally particularly in the form of a gel, ointment, lotion, suspension or patch delivery system with chemical enhancers such as dimethyl sulfoxide to either modify the skin structure or to increase the drug concentration in the transdermal patch (Junginger, et al. In "Drug Permeation Enhancement"; Hsieh, D. S., Eds., pp. 59-90 (Marcel Dekker, Inc. New York 1994, entirely incorporated herein by reference), or with oxidizing agents that enable the application of formulations containing proteins and peptides onto the skin (WO 98/53847), or applications of electric fields to create transient transport pathways such as electroporation, or to increase the mobility of charged drugs through the skin such as iontophoresis, or application of ultrasound such as sonophoresis (U.S. Pat. Nos. 4,309,989 and 4,767,402) (the above publications and patents being entirely incorporated herein by reference).

Pulmonary/Nasal Administration

For pulmonary administration, preferably at least one RELP Ig derived protein or specified portion or variant composition is delivered in a particle size effective for reaching the lower airways of the lung or sinuses. According to the invention, at least one RELP Ig derived protein or specified portion or variant can be delivered by any of a variety of inhalation or nasal devices known in the art for administration of a therapeutic agent by inhalation. These devices capable of depositing aerosolized formulations in the sinus cavity or alveoli of a patient include metered dose inhalers, nebulizers, dry powder generators, sprayers, and the like. Other devices suitable for directing the pulmonary or nasal administration of Ig derived

protein or specified portion or variants are also known in the art. All such devices can use of formulations suitable for the administration for the dispensing of Ig derived protein or specified portion or variant in an aerosol. Such aerosols can be comprised of either solutions (both aqueous and non aqueous) or solid particles. Metered dose inhalers like the Ventolin® metered dose inhaler, typically use a propellant gas and require actuation during inspiration (See, e.g., WO 94/16970, WO 98/35888). Dry powder inhalers like Turbuhaler™ (Astra), Rotahaler® (Glaxo), Diskus® (Glaxo), Spiros™ inhaler (Dura), devices marketed by Inhale Therapeutics, and the Spinhaler® powder inhaler (Fisons), use breath-actuation of a mixed powder (US 4668218 Astra, EP 237507 Astra, WO 97/25086 Glaxo, WO 94/08552 Dura, US 10 5458135 Inhale, WO 94/06498 Fisons, entirely incorporated herein by reference). Nebulizers like AERx™ Aradigm, the Ultravent® nebulizer (Mallinckrodt), and the Acorn II® nebulizer (Marquest Medical Products) (US 5404871 Aradigm, WO 97/22376), the above references entirely incorporated herein by reference, produce aerosols from solutions, while metered dose inhalers, dry powder inhalers, etc. generate small particle aerosols. These specific examples of 15 commercially available inhalation devices are intended to be a representative of specific devices suitable for the practice of this invention, and are not intended as limiting the scope of the invention. Preferably, a composition comprising at least one RELP Ig derived protein or specified portion or variant is delivered by a dry powder inhaler or a sprayer. There are a several desirable features of an inhalation device for administering at least one Ig derived 20 protein or specified portion or variant of the present invention. For example, delivery by the inhalation device is advantageously reliable, reproducible, and accurate. The inhalation device can optionally deliver small dry particles, e.g. less than about 10 μm , preferably about 1-5 μm , for good respirability.

25 Administration of RELP Ig derived protein or specified portion or variant Compositions as a Spray

A spray including RELP Ig derived protein or specified portion or variant composition protein can be produced by forcing a suspension or solution of at least one RELP Ig derived protein or specified portion or variant through a nozzle under pressure. The nozzle size and configuration, the applied pressure, and the liquid feed rate can be chosen to achieve the 30 desired output and particle size. An electrospray can be produced, for example, by an electric field in connection with a capillary or nozzle feed. Advantageously, particles of at least one RELP Ig derived protein or specified portion or variant composition protein delivered by a sprayer have a particle size less than about 10 μm , preferably in the range of about 1 μm to about 5 μm , and most preferably about 2 μm to about 3 μm .

RELB

Formulations of at least one RELP Ig derived protein or specified portion or variant composition protein suitable for use with a sprayer typically include Ig derived protein or specified portion or variant composition protein in an aqueous solution at a concentration of about 0.1 mg to about 100 mg of at least one RELP Ig derived protein or specified portion or variant composition protein per ml of solution or mg/gm, or any range or value therein, e.g., but not limited to, .1, .2, .3, .4, .5, .6, .7, .8, .9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/ml or mg/gm. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant, and, preferably, zinc. The formulation can also include an excipient or agent for stabilization of the Ig derived protein or specified portion or variant composition protein, such as a buffer, a reducing agent, a bulk protein, or a carbohydrate.

Bulk proteins useful in formulating Ig derived protein or specified portion or variant composition proteins include albumin, protamine, or the like. Typical carbohydrates useful in formulating Ig derived protein or specified portion or variant composition proteins include sucrose, mannitol, lactose, trehalose, glucose, or the like. The Ig derived protein or specified portion or variant composition protein formulation can also include a surfactant, which can reduce or prevent surface-induced aggregation of the Ig derived protein or specified portion or variant composition protein caused by atomization of the solution in forming an aerosol.

Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitol fatty acid esters. Amounts will generally range between 0.001 and 14% by weight of the formulation. Especially preferred surfactants for purposes of this invention are polyoxyethylene sorbitan monooleate, polysorbate 80, polysorbate 20, or the like. Additional agents known in the art for formulation of a protein such as RELP Ig derived proteins, or specified portions or variants, can also be included in the formulation.

Administration of RELP Ig derived protein or specified portion or variant compositions by a Nebulizer

Ig derived protein or specified portion or variant composition protein can be administered by a nebulizer, such as jet nebulizer or an ultrasonic nebulizer. Typically, in a jet nebulizer, a compressed air source is used to create a high-velocity air jet through an orifice. As the gas expands beyond the nozzle, a low-pressure region is created, which draws a solution of Ig derived protein or specified portion or variant composition protein through a capillary tube connected to a liquid reservoir. The liquid stream from the capillary tube is sheared into unstable filaments and droplets as it exits the tube, creating the aerosol. A range

of configurations, flow rates, and baffle types can be employed to achieve the desired performance characteristics from a given jet nebulizer. In an ultrasonic nebulizer, high-frequency electrical energy is used to create vibrational, mechanical energy, typically employing a piezoelectric transducer. This energy is transmitted to the formulation of Ig derived protein or specified portion or variant composition protein either directly or through a coupling fluid, creating an aerosol including the Ig derived protein or specified portion or variant composition protein. Advantageously, particles of Ig derived protein or specified portion or variant composition protein delivered by a nebulizer have a particle size less than about 10 μm , preferably in the range of about 1 μm to about 5 μm , and most preferably about 2 μm to about 3 μm .

Formulations of at least one RELP Ig derived protein or specified portion or variant suitable for use with a nebulizer, either jet or ultrasonic, typically include a concentration of about 0.1 mg to about 100 mg of at least one RELP Ig derived protein or specified portion or variant protein per ml of solution. The formulation can include agents such as an excipient, a buffer, an isotonicity agent, a preservative, a surfactant, and, preferably, zinc. The formulation can also include an excipient or agent for stabilization of the at least one RELP Ig derived protein or specified portion or variant composition protein, such as a buffer, a reducing agent, a bulk protein, or a carbohydrate. Bulk proteins useful in formulating at least one RELP Ig derived protein or specified portion or variant composition proteins include albumin, protamine, or the like. Typical carbohydrates useful in formulating at least one RELP Ig derived protein or specified portion or variant include sucrose, mannitol, lactose, trehalose, glucose, or the like. The at least one RELP Ig derived protein or specified portion or variant formulation can also include a surfactant, which can reduce or prevent surface-induced aggregation of the at least one RELP Ig derived protein or specified portion or variant caused by atomization of the solution in forming an aerosol. Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbital fatty acid esters. Amounts will generally range between 0.001 and 4% by weight of the formulation. Especially preferred surfactants for purposes of this invention are polyoxyethylene sorbitan mono-oleate, polysorbate 80, polysorbate 20, or the like. Additional agents known in the art for formulation of a protein such as Ig derived protein or specified portion or variant protein can also be included in the formulation.

Administration of RELP Ig derived protein or specified portion or variant compositions By A Metered Dose Inhaler

In a metered dose inhaler (MDI), a propellant, at least one RELP Ig derived protein or

specified portion or variant, and any excipients or other additives are contained in a canister as a mixture including a liquefied compressed gas. Actuation of the metering valve releases the mixture as an aerosol, preferably containing particles in the size range of less than about 10 μm , preferably about 1 μm to about 5 μm , and most preferably about 2 μm to about 3 μm . The desired aerosol particle size can be obtained by employing a formulation of Ig derived protein or specified portion or variant composition protein produced by various methods known to those of skill in the art, including jet-milling, spray drying, critical point condensation, or the like. Preferred metered dose inhalers include those manufactured by 3M or Glaxo and employing a hydrofluorocarbon propellant.

Formulations of at least one RELP Ig derived protein or specified portion or variant for use with a metered-dose inhaler device will generally include a finely divided powder containing at least one RELP Ig derived protein or specified portion or variant as a suspension in a non-aqueous medium, for example, suspended in a propellant with the aid of a surfactant.

The propellant can be any conventional material employed for this purpose, such as chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol and 1,1,1,2-tetrafluoroethane, HFA-134a (hydrofluroalkane-134a), HFA-227 (hydrofluroalkane-227), or the like. Preferably the propellant is a hydrofluorocarbon. The surfactant can be chosen to stabilize the at least one RELP Ig derived protein or specified portion or variant as a suspension in the propellant, to protect the active agent against chemical degradation, and the like. Suitable surfactants include sorbitan trioleate, soya lecithin, oleic acid, or the like. In some cases solution aerosols are preferred using solvents such as ethanol. Additional agents known in the art for formulation of a protein such as protein can also be included in the formulation.

One of ordinary skill in the art will recognize that the methods of the current invention can be achieved by pulmonary administration of at least one RELP Ig derived protein or specified portion or variant compositions via devices not described herein.

Oral Formulations and Administration

Formulations for oral rely on the co-administration of adjuvants (e.g., resorcinols and nonionic surfactants such as polyoxyethylene oleyl ether and n-hexadecylpolyethylene ether) to increase artificially the permeability of the intestinal walls, as well as the co-administration of enzymatic inhibitors (e.g., pancreatic trypsin inhibitors, diisopropylfluorophosphate (DFF) and trasylol) to inhibit enzymatic degradation. The active constituent compound of the solid-type dosage form for oral administration can be mixed with at least one additive, including

sucrose, lactose, cellulose, mannitol, trehalose, raffinose, maltitol, dextran, starches, agar, arginates, chitins, chitosans, pectins, gum tragacanth, gum arabic, gelatin, collagen, casein, albumin, synthetic or semisynthetic polymer, and glyceride. These dosage forms can also contain other type(s) of additives, e.g., inactive diluting agent, lubricant such as magnesium stearate, paraben, preserving agent such as sorbic acid, ascorbic acid, .alpha.-tocopherol, antioxidant such as cysteine, disintegrator, binder, thickener, buffering agent, sweetening agent, flavoring agent, perfuming agent, etc.

Tablets and pills can be further processed into enteric-coated preparations. The liquid preparations for oral administration include emulsion, syrup, elixir, suspension and solution preparations allowable for medical use. These preparations may contain inactive diluting agents ordinarily used in said field, e.g., water. Liposomes have also been described as drug delivery systems for insulin and heparin (U.S. Pat. No. 4,239,754). More recently, microspheres of artificial polymers of mixed amino acids (proteinoids) have been used to deliver pharmaceuticals (U.S. Pat. No. 4,925,673). Furthermore, carrier compounds described in U.S. Pat. No. 5,879,681 and U.S. Pat. No. 5,5,871,753 are used to deliver biologically active agents orally are known in the art.

Mucosal Formulations and Administration

For absorption through mucosal surfaces, compositions and methods of administering at least one RELP Ig derived protein or specified portion or variant include an emulsion comprising a plurality of submicron particles, a mucoadhesive macromolecule, a bioactive peptide, and an aqueous continuous phase, which promotes absorption through mucosal surfaces by achieving mucoadhesion of the emulsion particles (U.S. Pat. Nos. 5,514,670). Mucous surfaces suitable for application of the emulsions of the present invention can include corneal, conjunctival, buccal, sublingual, nasal, vaginal, pulmonary, stomachic, intestinal, and rectal routes of administration. Formulations for vaginal or rectal administration, e.g. suppositories, can contain as excipients, for example, polyalkyleneglycols, vaseline, cocoa butter, and the like. Formulations for intranasal administration can be solid and contain as excipients, for example, lactose or can be aqueous or oily solutions of nasal drops. For buccal administration excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like (U.S. Pat. Nos. 5,849,695).

Transdermal Formulations and Administration

For transdermal administration, the at least one RELP Ig derived protein or specified portion or variant is encapsulated in a delivery device such as a liposome or polymeric nanoparticles, microparticle, microcapsule, or microspheres (referred to collectively as

microparticles unless otherwise stated). A number of suitable devices are known, including microparticles made of synthetic polymers such as polyhydroxy acids such as polylactic acid, polyglycolic acid and copolymers thereof, polyorthoesters, polyanhydrides, and polyphosphazenes, and natural polymers such as collagen, polyamino acids, albumin and other proteins, alginate and other polysaccharides, and combinations thereof (U.S. Pat. Nos. 5,814,599).

Prolonged Administration and Formulations

It can be sometimes desirable to deliver the compounds of the present invention to the subject over prolonged periods of time, for example, for periods of one week to one year from a single administration. Various slow release, depot or implant dosage forms can be utilized. For example, a dosage form can contain a pharmaceutically acceptable non-toxic salt of the compounds that has a low degree of solubility in body fluids, for example, (a) an acid addition salt with a polybasic acid such as phosphoric acid, sulfuric acid, citric acid, tartaric acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalene mono- or di-sulfonic acids, polygalacturonic acid, and the like; (b) a salt with a polyvalent metal cation such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium and the like, or with an organic cation formed from e.g., N,N'-dibenzyl-ethylenediamine or ethylenediamine; or (c) combinations of (a) and (b) e.g. a zinc tannate salt. Additionally, the compounds of the present invention or, preferably, a relatively insoluble salt such as those just described, can be formulated in a gel, for example, an aluminum monostearate gel with, e.g. sesame oil, suitable for injection. Particularly preferred salts are zinc salts, zinc tannate salts, pamoate salts, and the like. Another type of slow release depot formulation for injection would contain the compound or salt dispersed for encapsulated in a slow degrading, non-toxic, non-antigenic polymer such as a polylactic acid/polyglycolic acid polymer for example as described in U.S. Pat. No. 3,773,919. The compounds or, preferably, relatively insoluble salts such as those described above can also be formulated in cholesterol matrix silastic pellets, particularly for use in animals. Additional slow release, depot or implant formulations, e.g. gas or liquid liposomes are known in the literature (U.S. Pat. Nos. 5,770,222 and "Sustained and Controlled Release Drug Delivery Systems", J. R. Robinson ed., Marcel Dekker, Inc., N.Y., 1978).

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Example 1: Cloning and sequencing of cDNAs

An EST that was abundantly expressed in mucinous ovarian tumor-derived libraries was identified. A full length cDNA insert, encoding for the predicted preprotein based on the 5 EST was acquired, cloned into the pSport vector, and verified by sequencing. Blasting with the human RELP cDNA in the NCBI EST data base yielded three highly homologous mouse sequences. The corresponding clones (IMAGE clone IDs 717371, 1079498 and 1096767) were acquired and sequenced. The putative mouse orthologue for RELP was cloned into the XbaI site in the pGEMA1bSVPA vector. Murine RELP and the nucleic acid that codes for it was 10 found to have 66% amino acid and 70% nucleotide sequence identity respectively to human RELP and its associated nucleic acid. Murine RELP was also found to have 43-45% similarity and 32%-37% identity to other mouse Reg sequences. The nucleic acid sequence of cDNA used to express it is shown in Fig. 1 (**Seq. ID No. 1**).

15 Example 2: Antibodies

A C-terminal RELP-derived peptide was synthesized (CAEMSSNNNFLTWSSNE **Seq. ID No. 5**), coupled to keyhole limpet hemocyanin, and used to immunize rabbits for production of polyclonal antibodies. The sera were tested for reactivity against the corresponding peptide with ELISA, and the positive batches were affinity-purified. The 20 purified antibody specifically detected the protein that has the peptide epitope in tissue sections. This was verified by complete abolishment of the signal if the corresponding peptide is added simultaneously with the antibody. In addition to this polyclonal antibody, which works well in immunohistochemistry, monoclonal antibodies able to detect the protein in its natural fold were produced. To produce monoclonal antibodies, a purified antigen, produced in 25 mammalian cells to ensure natural fold and posttranslational modifications, was generated. The antigen, RELP-IgG constant part fusion protein, was expressed in mouse myeloma cells, and the secreted protein was purified using the Fc part as bait. This purified antigen was recognized in Western blot by the C-terminal polyclonal antibody, and by five other anti-RELP peptide antibodies (Below, **Seq. ID No.6- Seq. ID No.11**). The antigen was used to 30 generate mouse monoclonal antibodies against RELP by selecting out of the positive clones those that produced antibodies that reacted against RELP instead of the IgG constant part.

Kits for the clinical identification of RELP can be readily fashioned employing these and similar antibodies. Such kits would include antibodies directed to RELP identification, appropriate indicator reagents (e.g., enzymes, labels, and the like), and (optionally) other

reagents useful in the clinical application of such a kit such as dilution buffers, stabilizers, and other materials typically used in such assays. The kits would be used to detect RELP in body fluids to screen or follow-up RELP expressing cancers, and to screen the presence of RELP protein in tissue samples.

5 **Seq. ID No. 6 :** CYGYFRKLRNWSDAELECQSYGNGA
 Seq. ID No. 7 : WIDGAMYLYRSWSGKSMGGNKHC
 Seq. ID No. 8 : CAEMSSNNNFLTWSSNE
 Seq. ID No. 9 : CAEMSSNNNFLTWSSNECNKRQHFLCKYR
10 **Seq. ID No. 10:** CEYISGYQRSQPIWIGLHDPPQKRQQWQ
 Seq. ID No. 11 : CQSYGNGAHLASILSLKEASTIA

Example 3: Double immunofluorescence staining

Tissue sections of normal duodenal mucosa were double stained with the polyclonal peptide antibody against RELP (1:30; 25 μ g/ml) and a monoclonal antibody against chromogranin A (1:5000; 0.2 μ g/ml Chemicon, Temecula, CA) followed by tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit immunoglobulins (DAKO) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Immunoglobulins (ICN/Cappel). For control stainings primary antibodies were replaced with the IgG fractions of normal rabbit and mouse sera.

20 The colocalization of RELP and chromogranin A indicates that the RELP-expressing cells in the duodenum belong to the neuroendocrine population.

Example 4: In situ hybridization

Formalin fixed paraffin embedded tissue samples were cut into 5-7 m μ thick sections, mounted on silane coated glass slides, and incubated at 37°C over night and at 65°C for 30 min before deparaffinating twice for 10 min in xylene. Thereafter the samples were rehydrated through a graded series of ethanol solutions (100 to 70%), and rinsed twice for 5 min in phosphate buffered saline (PBS pH 7.0), treated twice for 5 min with 0.1 mol/L glycine in PBS, permeabilized for 15 min with 0.3% Triton X-100 in PBS. The sections were treated with proteinase K (Finnzymes, Helsinki, Finland) treatment (μ g/ml, in TE buffer; 100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0) at 37°C for 30 min, postfixed in 3% paraformaldehyde in PBS at 4°C for 5 min and rinsed twice in PBS. Positive charges were blocked by soaking the slides in 0.25% (v/v) acetic anhydride, 100 mmol/L triethanolamine, pH 8.0, twice for 5 min. The slides were equilibrated in 4xSSC, 50% (v/v) deionized formamide at 37°C for 10 min.

Probes were prepared by ligating a PCR-amplified 0.4 kb RELP cDNA insert into the pCR-II vector using a TA cloning kit (Invitrogen, San Diego CA, USA). The templates for RELP antisense or sense RNA probes were generated by linearizing the appropriate vector construct (in 3' to 5' direction or 5' to 3' direction, respectively). An RNA Labeling Kit (Boehringer-Mannheim) was used to generate digoxigenin labeled RNA probes by in vitro transcription.

The hybridization was performed overnight at 45°C using a hybridization mixture containing 1xDenhart's solution (0.2g/L Ficoll Type 400, Pharmacia), 0.2g/L polyvinylpyrrolidone, 0.2g/L bovine serum albumin (fraction V; Sigma), 40% formamide, 10% dextran sulfate, 4xSSC, 10 mmol/L dithiothreitol, 1mg/mL yeast tRNA, 1 mg/mL herring sperm DNA and 300 ng/mL digoxigenin-labeled RNA probe. After hybridization, the tissue sections were washed at 37°C twice for 5 min in 2xSSC and once for 15 min in 60% formamide, 0.2xSSC, followed by two 5 minute rinses in 2xSSC at room temperature and two 10 minute washes in 100 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl. The signal detection was carried out using 1:250 alkaline phosphatase-conjugated sheep antidigoxigenin fab fragments (Boehringer Mannheim). The signal was visualized by incubating the sections with NBT/BCIP Stock Solution (Boehringer Mannheim) for 1.5 hours.

Small numbers of RELP-positive cells were seen in the gastric mucosa and in exocrine pancreas. In normal colon, RELP was localized in epithelial cells in the bottom of the crypts. A strong RELP mRNA signal was seen in the cytoplasm of selected cells in the duodenal mucosa while most of the epithelium was negative. In mucinous cancers from ovary, stomach, colon and breast the RELP mRNA was also detected in the epithelial cells. The visualization of the RELP-specific mRNA confirmed that the RELP protein was expressed by these cells.

Example 5: Immunohistochemistry

An affinity-purified polyclonal antibody against the C-terminal peptide of RELP was used for the immunohistochemical detection and localization of RELP. Four µm sections from formalin-fixed and paraffin embedded normal and tumor tissue, obtained from the archives of the Department of Pathology, University of Helsinki, were mounted on 3-aminopropyl-triethoxy-silane (APES, Sigma, St. Louis, MO, U.S.A) coated slides. The sections were deparaffinized and rehydrated in graded concentrations of ethanol and treated with methanolic peroxide (0.5% hydrogen peroxide in absolute methanol) for 30 minutes at room temperature to block the endogenous peroxidase activity. Antigen retrieval was done in a microwave oven twice for 5 minutes (650W). An Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA, U.S.A) was used for immunoperoxidase staining. The RELP antibody was used at an

optimal dilution of 1:2000. Both the biotinylated second antibody and the peroxidase-labeled avidin-biotin complex were incubated on the sections for 30 minutes. The dilutions were made in PBS (pH 7.2), and all incubations were carried out in a moist chamber at room temperature. Between the different staining steps the slides were rinsed three times with PBS. The
5 peroxidase staining was visualised with a 3-amino-9-ethylcarbazole (Sigma) solution (0.2 mg/ml in 0.05 M acetate buffer containing 0.03% hydrogen peroxide, pH 5.0) at room temperature for 15 minutes. Finally, the sections were lightly counterstained with Mayer's haematoxylin and mounted with aqueous mounting media (Aquamount, BDH). In control experiments the primary antibodies were replaced with the IgG fraction of normal rabbit serum
10 or the primary antibody was preabsorbed with the RELP peptide. These stainings indicated the presence of the RELP protein in a subset of duodenal epithelial cells, in a subset of stomach mucosal epithelial cells, in a subset of exocrine pancreatic ductal cells, in colon crypt bottom cells, in a subset of mammary ductal epithelial cells, and in the epithelial cells of benign and malignant mucinous tumors originating from ovary, stomach, colon, breast, and pancreas,
15 while the stroma remained completely negative. The abundant and uniform expression of RELP protein in the epithelial cells from mucinous tumors further supports the use of RELP as a tumor marker. As a secreted protein RELP can be measured from the serum or plasma. Moreover, anti-RELP antibodies might prove useful in detecting solitary tumor cells in tissue samples and cytologic specimen.
20

Example 6: Structure and nucleotide sequence of the gene

RELP cDNA comprises 1517 nucleotides, and the protein coding region is made up of 476 bp of nucleotides encoding a preprotein of 158 amino acids. The 5' untranslated and 3' untranslated regions contain 440 and 601 nucleotides respectively. The first methionine (nt
25 441-443) is preceded by a Kozaks' consensus translational start site. (Kozak sequence AAG before initiating methionine). A polyadenylation signal (AATAAA) is located 510 bp downstream of the termination codon. The gene structure of the protein was deduced by the analysis of genomic databases in the public domain. The missing base pairs flanking ends of the randomly ordered fragments of the genomic data base were acquired by sequencing these
30 areas of the physical genomic RELP sequence.

A human genomic PAC clone containing the genomic RELP sequence was obtained from GenomeSystemsInc (St.Louis, Missouri). NS3516 bacterial cells were transformed with the PAC plasmid containing a genomic insert of about 120kb. Plasmid DNA was isolated

using EndoFree Plasmid Maxi Kit (Qiagen, Germany). The genomic sequence was amplified by PCR using RELP-specific primers flanking the missing sequence data.

The primers used were as follows:

5	CAGCTGTGCTCCTGGATGGT Seq. ID No. 12
	TGGTCGGTACTTGCACAGGA Seq. ID No. 13
	CTCCTATTGCTGAGCTGCCT Seq. ID No. 14
	ATTCGTTGCTGCTCCAAGTT Seq. ID No. 15
	TTCCAGAAGCATGCGGCTG Seq. ID No. 16
10	ACAGGAAGTGTGGCGCTT Seq. ID No. 17
	ATGGCTTCCAGAACATGC Seq. ID No. 18
	CTATGGTCGGTACTTGCACA Seq. ID No. 19
	CTTGCTCTATGGTCGGTACT Seq. ID No. 20
	ACTGGGACCCTGGAGACACT Seq. ID No. 21
15	GAGACACTGAAGAACGGCAG Seq. ID No. 22
	AGACCCAGCTGTTCATAGG Seq. ID No. 23
	AATGGAGAGAGGGCAGAACAGG Seq. ID No. 24
	TGATATCATCATGAGACCCAGCT Seq. ID No. 25
	AGACAGTCATCCATTGCCCA Seq. ID No. 26
20	TGGGCAAATGGATGACTGTCT Seq. ID No. 27
	CTCTAGAACATCCAACAAACTC Seq. ID No. 28
	TGCCAGACCAGGATCTGTACA Seq. ID No. 29
	ATCCATATCGGCTGGCTTC Seq. ID No. 30
	CACTATGAAGAGAACGCCCT Seq. ID No. 31
	AAACACAACTGCTGCAGCGT Seq. ID No. 32
25	GAAGCCAGCCGATATGGAT Seq. ID No. 33
	TAGAGCTAGAACGCCACTACT Seq. ID No. 34
	TCCTGTGCAAGTACCGACCA Seq. ID No. 35
	CAGTAGTGGCTCTAGCTCT Seq. ID No. 36
	TCCTGGCACTATGAAGAG Seq. ID No. 37
30	GGTAGCAATATTGTAGAACATCC Seq. ID No. 38
	GTTCGTAGCACACTCCTGAT Seq. ID No. 39
	TATGGCTGCAGTCTGCGGT Seq. ID No. 40
	ACTAGAGTGGTCATGGGAAC Seq. ID No. 41
	GATTCCAGTTGCAAGGTAC Seq. ID No. 42

TACTGCTACTGCTGGGAAT **Seq. ID No. 43**

Amplified DNA fragments were subcloned into a TA vector and nucleotide sequences of the *relp* gene fragments were obtained by sequencing with vector-derived and *relp* specific primers. Comparison of genomic RELP DNA with the RELP cDNA sequence revealed that the transcribed regions are divided into seven exons separated by six introns and that all exon-intron junctions followed the GT-AG rule. The lengths of exons 1,2,3,4,5,6,7 are of 172, 174, 161, 98, 137, 106 and 669 bp respectively (Fig 4). It was determined that due to differential splicing exon 2 is not represented in all transcripts. The initiation of the first exon was deduced from the genomic sequence using the AG rule and the splice donor acceptor site consensus sequence location. Exons 1 to 3 encode the 5' untranslated region of 440nt (or 266 nt in the splice variants where the exon 2 is missing) and exon 7 the 3' untranslated region of 601 nt.

The promoter sequence of the *relp* gene was analyzed with the promoter analyzing program Genomatix (http://genomatix.gsf.de/mat_fam). An Ap-1 binding site and a cAMP responsive element are located at 15 respectively 44 base pairs upstream from transcriptional initiation site.

Example 7: Fluorescence in situ hybridization (FISH)

To determine the chromosomal localization of the *relp* gene, fluorescent in situ hybridization (FISH) was performed. A human genomic PAC clone containing the RELP gene was used as a probe to localize RELP in human chromosomes. The PAC plasmid was labeled with biotin-16-dUTP using nick translation. Slides with human interphase and metaphase nuclei were pretreated with 0.01 N HCl for 10 min at 37°C and 0.01 N HCl containing pepsin (20mg/ml) for 5 min at 37°C. After dehydration in graded ethanol, the slides were denatured in 70% formamide/2xSSC at 64°C. Hybridization was carried out at 37°C overnight. After hybridization, the slides were washed in 2xSSC for 1x5 min at 45°C, 0.1xSSC for 2x5 min at 45°C and in 4xSSC/0.2% Tween 1x5 min at room temperature, blocked in 5% BSA/4xSSC for 30 min at 37°C and in 4xSSC/0.2% Tween for 5min at 45°C. Hybridized probes were detected with avidin-conjugated FITC and the signals were amplified with biotinylated-anti-avidin antibodies. After washing at 45°C in 4xSSC/0.2% Tween for 3x5min the slides were counterstained with DAPI and mounted in an antifade solution.

Hybridization showed exclusive signals on chromosome 1 band p12-13.1.

Example 8: Dot blot and Northern blot analysis

Dot blot and Northern blot analyses were performed using Multiple Tissue Expression (MTE) Array and Multiple Tissue Northern (MTN) blot II and III (Clontech.). ^{32}P -labeled full length RELP cDNA was used as a probe. Labeling was done with the Multiprime DNA labeling system kit (Amersham Pharmacia Biotech). For autoradiography filters were exposed to Kodak Biomax MS film for 1-3 days. Dot blot analysis revealed RELP mRNA in tissues of the gastrointestinal tract, in the prostate, and in testis. Northern blot analysis demonstrated high expression of a 1.5-kb transcript in the duodenum, stomach, testis, and prostate. Significant expression was also seen in the jejunum, ileum, iloecum, appendix, descending colon and pancreas. No RELP expression was seen in thyroid, spinal cord, adrenal gland, bone marrow, spleen, thymus, ovary or blood leukocytes.

10 The above is the description of the normal tissue distribution of RELP.

In the cancers identified in the body of the specification above, RELP is expressed ectopically, meaning that it is expressed in cells which should not express it at all, where its expression is irrelevant, and is due to the regression of the level of differentiation. Thus, the presence of RELP beyond normal levels is seen at the level of the whole organism: the body produces too much RELP (measured in plasma), which indicates that there is a cancer in one of the organs known to develop RELP-positive tumors.

Example 9: Reverse transcription polymerase chain reaction (RT-PCR)

20 Reverse transcription and PCR amplification of RELP mRNA was performed by continuous RT-PCR using the Robust RT-PCR kit (Finnzymes, Espoo, Finland). One hundred ng of poly(A) RNA was reverse-transcribed into cDNA for one RT-PCR reaction. The primers used were as follows: sense:

25 CAGCTGTGCTCCTGGATGGT, Seq. ID No. 12

CTCCTATTGCTGAGCTGCCT Seq. ID No. 14

antisense: TGGTCGGTACTTGCACAGGA, Seq. ID No. 44

ATTCGTTGCTGCTCCAAGTT Seq. ID No. 45

Reverse transcription reaction was performed at 48°C for 30 min. Before PCR amplification, the samples were initially denatured at 95°C for 4 min. Cycling parameters were 30 as follows (30x): denaturation at 95°C for 30s, annealing at 60°C for 1 min, elongation at 72°C for 1 min and final extension at 72°C for 5 min.

Amplified products were analyzed by agarose electrophoresis and subcloned according to manufacturer's instructions into a vector of the TA cloning system (Invitrogen, San Diego). Nucleotide sequencing of the cloned PCR products were performed by the Thermo Sequenace

Kit (Amersham, Buckingshire, UK) and an ALF express sequenator (Pharmacia, Uppsala, Sweden). The procedure verified the transcription of RELP in duodenum, colon, stomach, and pancreas, and excluded the possibility that the Northern blot and Dot blot experiments should have detected RNA representing other reg proteins that are homologous to RELP.

5

Example 10: In vitro translation

A cDNA fragment containing the full length sequence of RELP cDNA was subcloned into the eukaryotic expression vector pCDNA 3 (Invitrogen, San Diego) under the T7 RNA polymerase promoter. The RELP protein was expressed using Rabbit Reticulocyte Lysate with Canine Pancreatic Microsomal Membranes (Promega, Madison, Wisconsin) in the presence of ³⁵S-methionine (Amersham International's Redivue L-35Smethionine, Amersham Pharmacia Biotech). Proteins obtained by in vitro translation were analyzed by SDS-PAGE (12%) gel electrophoresis and visualized by autoradiography. The translation resulted in a protein product with an apparent molecular weight of 18 kd as analysed by PAGE. This is in concordance with the calculated molecular weight of RELP (18.2kd). When the microsomal membrane fraction was added, the size of the protein product was reduced to 17kd, which is in concordance with the predicted structure of RELP, including an N-terminal cleavable 23 amino acid signal peptide.

20 Example 11: Enzyme Immunoassay (Prophetic)

Immunoassays are prepared for the RELP antigen. This is achievable since detection of 10 fmol/L is possible in competitive assays. Sensitivity of noncompetitive assay is determined by lower limit of detection of the label used: 1 to 2,000,000 Zeptomoles (10^{-21} moles). Tietz Fundamentals of Clinical Chemistry" 4th Edition, p143

To develop an Enzyme Immunoassay (EIA) procedure, antigen standards comprising a digest of colon tumor specimens (shown to contain the antigen by immunoperoxidase staining) are used. Human primary colon cancer specimens are pooled and homogenized in 10 volumes of 10 mM Tris buffer, pH 7.4, containing 0.2% (w/v) sodium deoxycholate at 4C. The homogenate is quickly brought to 37 C and the following reagents (final concentration) are added while stirring: 1 mM cysteine (Sigma), 1 mM EDTA (Sigma), and papain (0.8 unit/ml) (Boehringer-Mannheim, Indianapolis, Ind.). After 5 minutes, digestion is stopped by the addition of 5 mM iodoacetamide (Sigma). The homogenate is centrifuged at 100,000Xg for 1 hour at 4C, then extensively dialyzed against 10 mM Tris/0.9% NaCl solution buffer, pH 7.4,

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containing phenylmethysulfonyl fluoride and aminocaproic acid, each at 10 mM. The homogenate is frozen in small aliquots at a concentration of 0.5 mg of protein/ml.

The dose response curve that will be generated for the immunoassay procedure measuring RELP demonstrates linearity between antigen input of 100ng to 100 μ g/ml. For serum analysis, the range is 1ng to 1000ng/ml, since these samples are diluted 10-fold prior to assay.

Solid-phase preparations of the antibodies described in Example 2 are prepared using CNBr-activated Sepharose (Pharmacia). Microtiter plates (Nunc I Immunoplates; Grand Island Biological Co., Grand Island, N.Y.) are coated with the antibodies (200 μ l /well) in 50 mM carbonate-bicarbonate buffer, pH 9.6, for 18 hours at 4C. After removal of the antibody solution, residual protein binding sites on the plastic are blocked by the addition of 200 μ l of assay buffer [PBS containing 1% (v/v) rabbit serum and 1% (w/v) bovine albumin]. After 1 hour of incubation at room temperature, the coated plates are used immediately for the assay procedure.

To perform the assay, 200 μ l samples, diluted in assay buffer, are applied for 1-5 hours at 37C. After 3 washes using assay buffer, 200 μ l of the antibody covalently conjugated to horseradish peroxidase (Sigma, Type VI) is applied to each well for 1.5 hours at 37C. The conjugate is diluted to a concentration of 0.5 μ g of immunoglobulin per ml of PBS containing 10% (v/v) murine serum. Following a wash procedure as above, 200 μ l of substrate per well are applied for 0.5 hours at room temperature. Substrate solution contains 0.4 mg of o-phenylenediamine per ml of pH 5.0 citrate buffer and 0.003% hydrogen peroxide. The reaction is stopped by addition of 50 μ l of 2N sulfuric acid, and absorbance is monitored at 488 nM using an enzyme assay plate reader (Fisher Scientific Co., Pittsburgh, Pa.).

The percentage of bound enzyme conjugate is calculated by the formula:

$$\frac{(B - B_0)(B_t - B_0)}{B_t} \times 100$$

where B=absorbance of the sample, B_t=maximal absorbance, and B₀=absorbance of the blank. Each assay is performed in triplicate using a standard digest and 26-fold diluted serum samples diluted in assay buffer. Specificity of the immunoassay is examined by substituting various antibody reagents at the solid phase, including an antibody to CEA and nonimmune rabbit serum. Of the solid phase antibodies only antibody prepared according to Example 2 binds antigen at high dilutions.

Levels of serum RELP are detected for normal control subjects, patients with benign and malignant prostate diseases and patients with ovarian, stomach, colon, and breast cancer.

Sera obtained from apparently healthy individuals exhibits a mean value of approximately 90ng/ml of RELP/ml. Only 5% of the samples express serum antigen at 5 150ng/ml or above, and this value is chosen as the cutoff for elevated serum levels.

Sera from patients with benign disease of the colon exhibit a mean RELP value of 160ng/ml (Table IV). The incidence of values above 200 ng/ml is 5%. Patients with colon cancer (with evidence of disease) exhibit a wide range of circulating levels of RELP with a mean value above 160ng/ml.

10 Sera obtained from patients with cancers corresponding to those described above are also evaluated. The incidence of elevated RELP values is 90%. Mean serum values from the group with cancer are significantly higher than control levels (about 250% higher).

15 Using a limited number of postoperative colon cancer patients with primary localized disease, a significant decrease in serum RELP occurs. These data indicate a relationship between serum RELP levels and tumor load. Such measurements are thus valuable for patient monitoring.

20 **Example 12: Cloning and Expression of RELP Ig derived protein in Mammalian Cells**

A typical mammalian expression vector contains at least one promoter element, which mediates the initiation of transcription of mRNA, the Ig derived protein or specified portion or variant coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention 25 include, for example, vectors such as pIRES1neo, pRetro-Off, pRetro-On, PLXSN, or pLNCX (Clonetech Labs, Palo Alto, CA), pcDNA3.1 (+/-), pcDNA/Zeo (+/-) or pcDNA3.1/Hygro (+/-) (Invitrogen), PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 30 7 and CV 1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded Ig derived protein or specified portion or variant. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy, et al., Biochem. J. 227:277-279 (1991); Bebbington, et al., Bio/Technology 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of Ig derived protein or specified portion or variants.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen, et al., Molec. Cell. Biol. 5:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart, et al., Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of RELP Ig derived protein or specified portion or variant. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (e.g., alpha minus MEM, Life Technologies, Gaithersburg, MD) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., F. W. Alt, et al., J. Biol. Chem. 253:1357-1370 (1978); J. L. Hamlin and C. Ma, Biochem. et Biophys. Acta 1097:107-143 (1990); and M. J. Page and M. A. Sydenham, Biotechnology 9:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach can be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained that

contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen, et al., Molec. Cell. Biol. 5:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of 5 human cytomegalovirus (CMV) (Boshart, et al., Cell 41:521-530 (1985)). Downstream of the promoter are BamHI, XbaI, and Asp718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human b-actin promoter, the SV40 early or late promoters or 10 the long terminal repeats from other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the RELP protein in a regulated way in mammalian cells (M. Gossen, and H. Bujard, Proc. Natl. Acad. Sci. USA 89: 5547-5551 (1992)). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines 15 carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with restriction enzymes and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated 20 from a 1% agarose gel.

The DNA sequence encoding the complete RELP Ig derived protein or specified portion or variant is used, corresponding to HC and LC variable regions of a RELP Ig derived protein of the present invention, according to known method steps. Isolated nucleic acid 25 encoding a suitable human constant region (i.e., HC and LC regions) is also used in this construct (e.g., as provided in vector p1351).

The isolated variable and constant region encoding DNA and the dephosphorylated vector are then ligated with T4 DNA ligase. E. coli HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary (CHO) cells lacking an active DHFR gene are used for 30 transfection. 5 µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSV2-neo using lipofectin. The plasmid pSV2neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 µg/ml

G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 g /ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 5 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 mM, 2 mM, 5 mM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained that grow at a concentration of 100 - 200 mM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reverse phase HPLC analysis.
10

15 **Example 13: Generation of High Affinity Human IgG Monoclonal Ig derived proteins Reactive With Human RELP protein Using Transgenic Mice**

Summary

Transgenic mice have been used that contain human heavy and light chain immunoglobulin genes to generate high affinity, completely human, monoclonal Ig derived 20 proteins that can be used therapeutically to inhibit the action of RELP protein for the treatment of one or more RELP protein-mediated disease. (CBA/J x C57/BL6/J) F₂ hybrid mice containing human variable and constant region Ig derived protein transgenes for both heavy and light chains are immunized with human recombinant RELP protein (Taylor et al., Intl. Immunol. 6:579-591 (1993); Lonberg, et al., Nature 368:856-859 (1994); Neuberger, M., Nature Biotech. 14:826 (1996); Fishwild, et al., Nature Biotechnology 14:845-851 (1996)). Several fusions yielded one or more panels of completely human RELP protein reactive IgG 25 monoclonal Ig derived proteins. The completely human anti-RELP Ig derived proteins are further characterized. All are IgG1κ. Such Ig derived proteins are found to have affinity constants somewhere between 1x10⁹ and 9x10¹². The unexpectedly high affinities of these 30 fully human monoclonal Ig derived proteins make them suitable candidates for therapeutic applications in RELP protein related diseases, pathologies or disorders.

Abbreviations

BSA - bovine serum albumin
CO₂ - carbon dioxide
35 DMSO - dimethyl sulfoxide
EIA - enzyme immunoassay
FBS - fetal bovine serum
H₂O₂ - hydrogen peroxide
HRP - horseradish peroxidase\\
CEN0285

ID - interadermal
Ig - immunoglobulin
RELP protein - REG-Like Protein
IP - intraperitoneal
5 IV - intravenous
Mab - monoclonal Ig derived protein
OD - optical density
OPD - o-Phenylenediamine dihydrochloride
PEG - polyethylene glycol
10 PSA - penicillin, streptomycin, amphotericin
RT - room temperature
SQ - subcutaneous
v/v - volume per volume
w/v - weight per volume
15

Materials and Methods

Animals

Transgenic mice that can express human Ig derived proteins are known in the art (and are commercially available (e.g., from GenPharm International, San Jose, CA; Abgenix, 20 Freemont, CA, and others) that express human immunoglobulins but not mouse IgM or Ig κ .

For example, such transgenic mice contain human sequence transgenes that undergo $V(D)J$ joining, heavy-chain class switching, and somatic mutation to generate a repertoire of human sequence immunoglobulins (Lonberg, et al., Nature 368:856-859 (1994)). The light chain transgene can be derived, e.g., in part from a yeast artificial chromosome clone that includes nearly half of the germline human $V\kappa$ region. In addition, the heavy-chain transgene can encode both human μ and human $\gamma 1$ (Fishwild, et al., Nature Biotechnology 14:845-851 (1996)) and/or $\gamma 3$ constant regions. Mice derived from appropriate genotypic lineages can be used in the immunization and fusion processes to generate fully human monoclonal Ig derived proteins to RELP protein.

30 Immunization

One or more immunization schedules can be used to generate the anti-RELP protein human hybridomas. The first several fusions can be performed after the following exemplary immunization protocol, but other similar known protocols can be used. Several 14-20 week old female and/or surgically castrated transgenic male mice are immunized IP and/or ID with 35 1-1000 μ g of recombinant human RELP protein emulsified with an equal volume of TITERMAX or complete Freund's adjuvant in a final volume of 100-400 μ L (e.g., 200). Each mouse can also optionally receive 1-10 μ g in 100 μ L physiological saline at each of 2 SQ sites. The mice can then be immunized 1-7, 5-12, 10-18, 17-25 and/or 21-34 days later IP (1-400 μ g) and SQ (1-400 μ g x 2) with RELP protein emulsified with an equal volume of

CEN0285

TITERMAX or incomplete Freund's adjuvant. Mice can be bled 12-25 and 25-40 days later by retro-orbital puncture without anti-coagulant. The blood is then allowed to clot at RT for one hour and the serum is collected and titered using an RELP protein EIA assay according to known methods. Fusions are performed when repeated injections do not cause titers to increase. At that time, the mice can be given a final IV booster injection of 1-400 µg RELP protein diluted in 100 µL physiological saline. Three days later, the mice can be euthanized by cervical dislocation and the spleens removed aseptically and immersed in 10 mL of cold phosphate buffered saline (PBS) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (PSA). The splenocytes are harvested by steriley perfusing the spleen with PSA-PBS. The cells are washed once in cold PSA-PBS, counted using Trypan blue dye exclusion and resuspended in RPMI 1640 media containing 25 mM Hepes.

Cell Fusion

Fusion can be carried out at a 1:1 to 1:10 ratio of murine myeloma cells to viable spleen cells according to known methods, e.g., as known in the art. As a non-limiting example, spleen cells and myeloma cells can be pelleted together. The pellet can then be slowly resuspended, over 30 seconds, in 1 mL of 50% (w/v) PEG/PBS solution (PEG molecular weight 1,450, Sigma) at 37 °C. The fusion can then be stopped by slowly adding 10.5 mL of RPMI 1640 medium containing 25 mM Hepes (37 °C) over 1 minute. The fused cells are centrifuged for 5 minutes at 500-1500 rpm. The cells are then resuspended in HAT medium (RPMI 1640 medium containing 25 mM Hepes, 10% Fetal Clone I serum (Hyclone), 1 mM sodium pyruvate, 4 mM L-glutamine, 10 µg/mL gentamicin, 2.5% Origin culturing supplement (Fisher), 10% 653-conditioned RPMI 1640/Hepes media, 50 µM 2-mercaptoethanol, 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine) and then plated at 200 µL/well in fifteen 96-well flat bottom tissue culture plates. The plates are then placed in a humidified 37 °C incubator containing 5% CO₂ and 95% air for 7-10 days.

Detection of Human IgG Anti-RELP Ig derived proteins in Mouse Serum

Solid phase EIA's can be used to screen mouse sera for human IgG Ig derived proteins specific for human RELP protein. Briefly, plates can be coated with RELP protein at 2 µg/mL in PBS overnight. After washing in 0.15M saline containing 0.02% (v/v) Tween 20, the wells can be blocked with 1% (w/v) BSA in PBS, 200 µL/well for 1 hour at RT. Plates are used immediately or frozen at -20 °C for future use. Mouse serum dilutions are incubated on the RELP protein coated plates at 50 µL/well at RT for 1 hour. The plates are washed and then probed with 50 µL/well HRP-labeled goat anti-human IgG, Fc specific diluted 1:30,000 in 1% BSA-PBS for 1 hour at RT. The plates can again be washed and 100 µL/well of the

citrate-phosphate substrate solution (0.1M citric acid and 0.2M sodium phosphate, 0.01% H₂O₂ and 1 mg/mL OPD) is added for 15 minutes at RT. Stop solution (4N sulfuric acid) is then added at 25 µL/well and the OD's are read at 490 nm via an automated plate spectrophotometer.

5 **Detection of Completely Human Immunoglobulins in Hybridoma Supernates**

Growth positive hybridomas secreting fully human immunoglobulins can be detected using a suitable EIA. Briefly, 96 well pop-out plates (VWR, 610744) can be coated with 10 µg/mL goat anti-human IgG Fc in sodium carbonate buffer overnight at 4 °C. The plates are washed and blocked with 1% BSA-PBS for one hour at 37°C and used immediately or frozen at -20 °C. Undiluted hybridoma supernatants are incubated on the plates for one hour at 37°C. The plates are washed and probed with HRP labeled goat anti-human kappa diluted 1:10,000 in 1% BSA-PBS for one hour at 37°C. The plates are then incubated with substrate solution as described above.

10 **Determination of Fully Human Anti-RELP protein Reactivity**

15 Hybridomas, as above, can be simultaneously assayed for reactivity to RELP protein using a suitable RIA or other assay. For example, supernatants are incubated on goat anti-human IgG Fc plates as above, washed and then probed with radiolabeled RELP protein with appropriate counts per well for 1 hour at RT. The wells are washed twice with PBS and bound radiolabeled RELP protein is quantitated using a suitable counter.

20 Human IgG1κ anti-RELP protein secreting hybridomas can be expanded in cell culture and serially subcloned by limiting dilution. The resulting clonal populations can be expanded and cryopreserved in freezing medium (95% FBS, 5% DMSO) and stored in liquid nitrogen.

Isotyping

25 Isotype determination of the Ig derived proteins can be accomplished using an EIA in a format similar to that used to screen the mouse immune sera for specific titers. RELP protein can be coated on 96- well plates as described above and purified Ig derived protein at 2 µg/mL can be incubated on the plate for one hour at RT. The plate is washed and probed with HRP labeled goat anti-human IgG₁ or HRP labeled goat anti-human IgG₃ diluted at 1:4000 in 1% BSA-PBS for one hour at RT. The plate is again washed and incubated with substrate solution as described above.

30 Binding Kinetics of Human Anti-Human RELP Ig derived proteins With Human RELP protein

Binding characteristics for Ig derived proteins can be suitably assessed using an RELP protein capture EIA and BIACore technology, for example. Graded concentrations of purified

human RELP Ig derived proteins can be assessed for binding to EIA plates coated with 2 µg/mL of RELP protein in assays as described above. The OD's can be then presented as semi-log plots showing relative binding efficiencies.

Quantitative binding constants can be obtained, e.g., as follows, or by any other known
5 suitable method. A BIACore CM-5 (carboxymethyl) chip is placed in a BIACore 2000 unit.
HBS buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v P20 surfactant, pH 7.4)
is flowed over a flow cell of the chip at 5 µL/minute until a stable baseline is obtained. A
solution (100 µL) of 15 mg of EDC (N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide
hydrochloride) in 200 µL water is added to 100 µL of a solution of 2.3 mg of NHS
10 (N-hydroxysuccinimide) in 200 µL water. Forty (40) µL of the resulting solution is injected
onto the chip. Six µL of a solution of human RELP protein (15 µg/mL in 10 mM sodium
acetate, pH 4.8) is injected onto the chip, resulting in an increase of ca. 500 RU. The buffer is
changed to TBS/Ca/Mg/BSA running buffer (20 mM Tris, 0.15 M sodium chloride, 2 mM
calcium chloride, 2 mM magnesium acetate, 0.5% Triton X-100, 25 µg/mL BSA, pH 7.4) and
15 flowed over the chip overnight to equilibrate it and to hydrolyze or cap any unreacted
succinimide esters.

Ig derived proteins are dissolved in the running buffer at 33.33, 16.67, 8.33, and 4.17
nM. The flow rate is adjusted to 30 µL/min and the instrument temperature to 25 °C. Two
flow cells are used for the kinetic runs, one on which RELP protein had been immobilized
20 (sample) and a second, underivatized flow cell (blank). 120 µL of each Ig derived protein
concentration is injected over the flow cells at 30 µL/min (association phase) followed by an
uninterrupted 360 seconds of buffer flow (dissociation phase). The surface of the chip is
regenerated (REG-Like Protein /Ig derived protein complex dissociated) by two sequential
injections of 30 µL each of 2 M guanidine thiocyanate.

25 Analysis of the data is done using BIA evaluation 3.0 or CLAMP 2.0, as known in the
art. For each Ig derived protein concentration the blank sensogram is subtracted from the
sample sensogram. A global fit is done for both dissociation (k_d , sec⁻¹) and association (k_a ,
mol⁻¹ sec⁻¹) and the dissociation constant (K_D , mol) calculated (k_d/k_a). Where the Ig derived
protein affinity is high enough that the RUs of Ig derived protein captured are >100, additional
30 dilutions of the Ig derived protein are run.

Results and Discussion

Generation of Anti-Human RELP protein Monoclonal Ig derived proteins

Several fusions are performed and each fusion is seeded in 15 plates (1440 wells/fusion) that yield several dozen Ig derived proteins specific for human RELP protein. Of

these, some are found to consist of a combination of human and mouse Ig chains. The remaining hybridomas secret anti-RELP Ig derived proteins consisting solely of human heavy and light chains. Of the human hybridomas all are expected to be IgG1κ.

Binding Kinetics of Human Anti-Human RELP Ig derived proteins

5 ELISA analysis confirms that purified Ig derived protein from most or all of these hybridomas bind RELP protein in a concentration-dependent manner. Figures 1-2 show the results of the relative binding efficiency of these Ig derived proteins. In this case, the avidity of the Ig derived protein for its cognate antigen (epitope) is measured. It should be noted that binding RELP protein directly to the EIA plate can cause denaturation of the protein and the
10 apparent binding affinities cannot be reflective of binding to undenatured protein. Fifty percent binding is found over a range of concentrations.

Quantitative binding constants are obtained using BIACore analysis of the human Ig derived proteins and reveals that several of the human monoclonal Ig derived proteins are very high affinity with K_D in the range of 1×10^{-9} to 7×10^{-12} .

15 Conclusions

Several fusions are performed utilizing splenocytes from hybrid mice containing human variable and constant region Ig derived protein transgenes that are immunized with human RELP protein. A set of several completely human RELP protein reactive IgG monoclonal Ig derived proteins of the IgG1κ isotype are generated. The completely human
20 anti-RELP Ig derived proteins are further characterized. Several of generated Ig derived proteins have affinity constants between 1×10^9 and 9×10^{12} . The unexpectedly high affinities of these fully human monoclonal Ig derived proteins make them suitable for therapeutic applications in RELP protein-dependent diseases, pathologies or related conditions.

25

It will be clear that the invention can be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

CEN0285